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REPORT OF THE VALIDATION STUDY OF THE MCTT HUMAN CORNEAL-LIKE EPITHELIUM EYE IRRITATION TEST MODEL AND REPORT OF THE VALIDATION PEER-REVIEW

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REPORT OF THE VALIDATION STUDY OF THE MCTT HUMAN CORNEAL-LIKE EPITHELIUM EYE IRRITATION TEST MODEL AND REPORT OF THE VALIDATION PEER-REVIEW

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Environment Directorate
ORGANISATION FOR ECONOMIC COOPERATION AND DEVELOPMENT
Paris 2019

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FOREWORD

This document contains in Annex 1: The report of the validation study of the MCTTTM human Cornea-like Epithelium (hCE) Eye Irritation Test (EIT), prepared by the lead country, Korea, and in Annex 2: The peer-review report of the validation, coordinated by the Korean Centre for the Validation of Alternative Methods (KoCVAM).

The two reports have been circulated to the Expert Group and the Working Group of the National Coordinators of the Test Guidelines Programme (WNT) for review on the occasion of the first commenting round on the draft updated Test Guideline 492 (TG 492).

In April 2019, the Working Group of the National Coordinators of the Test Guidelines Programme approved the updated TG 492, now including the MCTT test method, and endorsed the validation and peer-review reports presented in this document.

ANNEX 1: The report of the validation study of the MCTT™ human Cornea-like Epithelium (hCE) Eye Irritation Test (EIT)

MCTT HCE™ EIT Validation Report

Me-Too Validation study for *in vitro* eye irritation test with 3D-reconstructed human cornea epithelium, MCTT HCE™

25th July 2018

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Acronyms and Abbreviations

BLR	Between-Laboratory Reproducibility
CAS	Chemical Abstracts Service
CST	Chemical Selection Team
DPBS	Dulbecco's Phosphate Buffered Saline
ECETOC	European Centre for Ecotoxicology and Toxicology of Chemicals
EIT	Eye Irritation Test
ET_{50}	Effective Time 50
EURL-ECVAM	European Union Reference Laboratory–European Centre for the Validation of Alternative Methods
GHS	Globally Harmonized System of Classification and Labelling of Chemicals
GLP	Good Laboratory Practice
KoCVAM	Korean Center for the Validation of Alternative Methods
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
OD	Optical Density
OECD	Organization for Economic Cooperation and Development
PS	Performance Standards
QA	Quality Assurance
QC	Quality Control
RhCE	Reconstructed human Corneal Epithelium
ROC	Receiver Operating Characteristic
SD	Standard Deviation
SOPs	Standard Operating Procedures

TG	Test Guideline
VMT	Validation Management Team
VRM	Validated Reference Method
WLR	Within-Laboratory Reproducibility
WNT	Working Group of the National Coordinators of the Test Guidelines Programme
WST-1	Water soluble tetrazolium salt-1 [2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] (CAS RN 150849-52-8)

Definitions

Accuracy: The closeness of agreement between test method results and accepted reference values. It is a measure of test method performance and one aspect of relevance.

ET_{50}: The time of exposure to 0.3% Triton X-100 estimated to decrease the viability by 50%

Lead laboratory: The laboratory responsible for training other participating facilities involved in standardization, optimization, and validation of a test method.

Project plan: A validation study plan designed to help participants understand the validation study by providing essential information and describing responsibilities and duties of each participating party.

Protocol: A test plan that clearly details each step of a validation method and provides criteria and a process to prepare reagents, supplies, and tools to generate test data.

Reference chemicals: Chemicals that have already been validated in other test systems and species and can be selected for use in the validation process.

Relevance: Description of the relationship between the effect of interest if a test method and whether it is meaningful and useful for a particular purpose. It is the extent to which a test correctly measures or predicts biological effects of interest.

Reliability: The extent to which a test method can be performed reproducibly within and between laboratories over time, when performed using the same protocol.

Reproducibility: The closeness of agreement between test method results using the same chemicals.

Run: A run consists of one or more test chemicals tested concurrently with a negative control and positive control.

Sensitivity: The percentage of positive chemicals correctly classified by a test method.

Specificity: The percentage of negative chemicals correctly classified by a test method.

Standard Operating Procedures (SOPs): A document that describes specific tests and the process of laboratory operation.

Transferability: The extent to which an independent testing facility can accurately and reliably perform a test procedure.

Validation: A process to demonstrate the reliability and relevance of an alternative test method.

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I. Goal Statement

The MCTT HCE™ Eye Irritation Test (EIT) validation study was intended to assess the within- and between-laboratory reproducibility as well as the predictive capacity of the MCTT HCE™ EIT in accordance with the performance standards in OECD GD 216 (OECD, 2015a).

II. Objective

The OECD Working Group of the National Coordinators of the Test Guidelines Programme (WNT) adopted a new *in vitro* test, OECD TG 492, to assess eye irritation using a reconstructed human cornea-like epithelium (RhCE) tissue model in July 2015 (OECD, 2015c). At the time of the original adoption, the only available validated reference method (VRM) was EpiOcular™ Eye Irritation Test (EIT) (OECD, 2015c). Then, me-too eye irritation tests using SkinEthic HCE™ EIT and LabCyte CORNEA-MODEL/24 EIT have been developed, and validation studies were completed. In June 2018, the SkinEthic HCE™ EIT was included in OECD TG 492 as VRM2 and LabCyte Cornea Model EIT, as Me-too test method (OECD, 2018b). The MCTT HCE™ EIT is a new *in vitro* test method for evaluating eye irritation using a new RhCE manufactured with primary human corneal cells (Jung *et al.*, 2011). Protocol optimization and pre-validation of the MCTT HCE™ EIT were completed in 2016 (Jang *et al.*, 2015; Yang *et al.*, 2017). The purpose of this validation study was to evaluate the reproducibility of within-laboratory and between-laboratory, and the predictive capacity (sensitivity, specificity, and accuracy) for ensuring that the MCTT HCE™ EIT meets the standard for a me-too test stated in OECD GD 216. This validation study was carried out in accordance with the OECD GD 216 performance standards published in July 2015 (revised in Aug. 2017) using 30 reference chemicals (OECD, 2015a). The validation study was conducted according to the modular approach as described in OECD Guidance Document 34 (OECD, 2005). To further evaluate the predictive capacity of MCTT HCE™ EIT, two reference chemicals newly adopted in the revised OECD GD 216 were tested and the data for a total of 141 reference chemicals that were compatible with the current protocol were collected from previous studies and analyzed.

III. Background

Since the EU ban on animal testing for cosmetics in 2013, more than 27 countries around the world including the Republic of Korea have legally prohibited animal experiments for the development of cosmetics. Accordingly, several *in vitro* tests have been developed to replace the TG 405 Draize eye irritation test, which has been used to evaluate the eye irritation potency of chemical chemicals, and the following test guidelines were approved as OECD TGs:

TG 437: Bovine Corneal Opacity and Permeability test method for identifying i) chemicals inducing serious eye damage and ii) chemicals not requiring classification for eye irritation or serious eye damage (OECD, 2017b)

TG 438: Isolated Chicken Eye test method for identifying i) chemicals inducing serious eye damage and ii) chemicals not requiring classification for eye irritation or serious eye damage (OECD, 2018a)

TG 460: Fluorescein Leakage test method for identifying ocular corrosives and severe irritants (OECD, 2012)

TG 491: Short Time Exposure in vitro test method for identifying i) chemicals inducing serious eye damage and ii) chemicals not requiring classification for eye irritation or serious eye damage (OECD, 2015b)

TG 492: Reconstructed human Cornea-like Epithelium (RhCE) test method for identifying chemicals not requiring classification and labeling for eye irritation or serious eye damage in accordance with UN GHS (OECD, 2018b)

The RhCE model is capable of distinguishing between irritants and non-irritants, and it can also be used in combination with other replacement methods to enable further classification of the hazards on eyes in the framework of tiered testing approach (Scott *et al.*, 2010).

The MCTT HCE™ model is a newly developed RhCE model, which is reconstructed using primary cultured human corneal epithelial cells from residual limbus tissues after corneal transplantation (Jung *et al.*, 2011). Therefore, its morphological microstructure and biomarker expressions are presumed to be similar to those of actual human corneal epithelium (Jung *et al.*, 2011). Studies have been conducted to develop new *in vitro* assays for evaluating eye irritation using MCTT HCE™, and the final protocol for MCTT HCE™ EIT was completed with several protocol refinements through pre-validation (Yang *et al.*, 2017) and the current validation study. The utility of the MCTT HCE™ EIT has been demonstrated in several papers published in international journals regarding assessing eye hazard and studying corneal physiology. (Choi *et al.*, 2015; Jang *et al.*, 2015; Lee *et al.*, 2018; Lee *et al.*, 2017; Yun *et al.*, 2016).

This validation study was conducted in accordance with the ~~by the~~ OECD GD 216 performance standards (OECD, 2015a) under the steering of a validation management team since the MCTT HCE™ EIT is based on a similar principle to OECD TG 492(OECD, 2018b).

IV. Reconstructed Human Corneal Epithelial Model

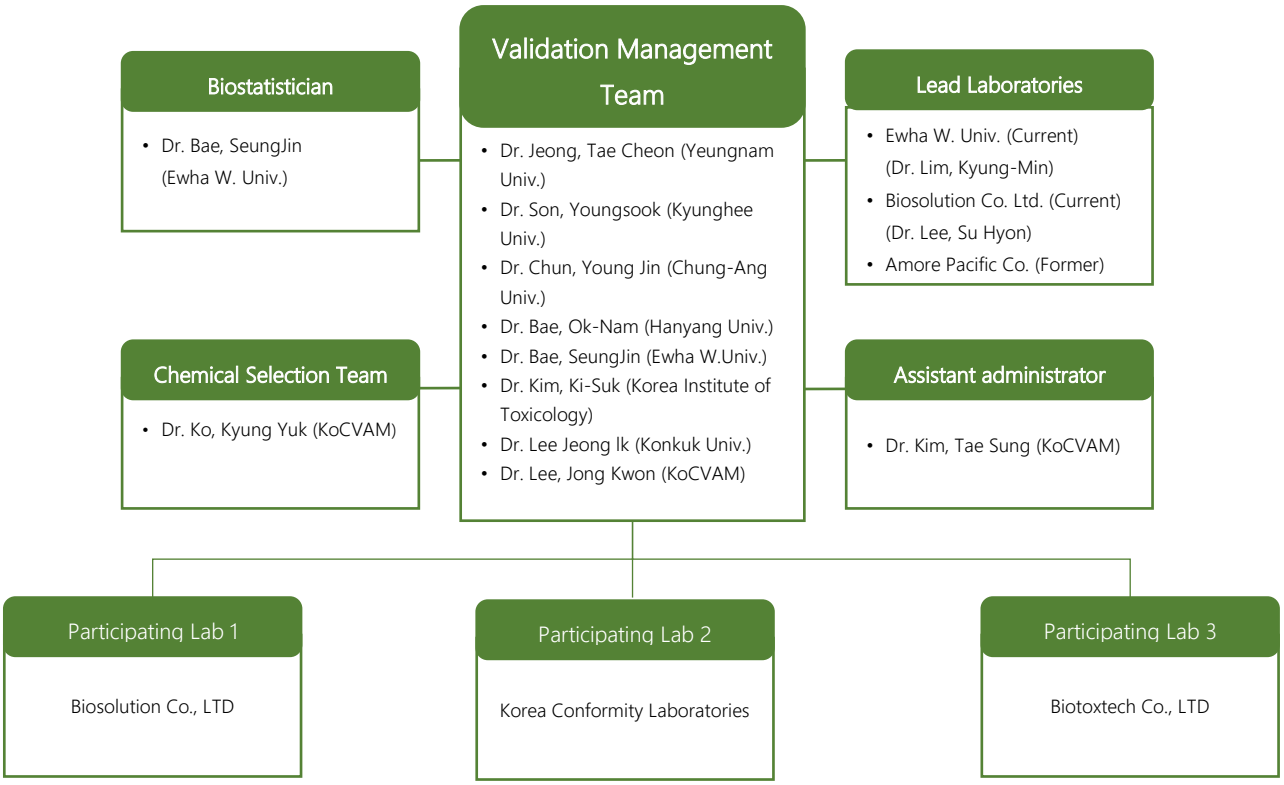
1. MCTT HCE™ EIT Model

The MCTT HCE™, a multilayered (≥ 3 layers) corneal epithelium model composed of basal cells, wing cells, and squamous cells, is produced by Biosolution Co., Korea. The MCTT HCE™ model is prepared from human corneal epithelial cells in residual limbus tissues remaining after corneal transplantation under the approval of Chung-Ang University Hospital IRB (#10-049-07-13). In order to reconstruct the MCTT HCE™ model, cultured corneal epithelial cells were transferred to the inner well of Millicell® (Millipore, Burlington, MA, USA) with a diameter of 12 mm, and cultivated on the air-

liquid interface for 7 days to regenerate stratified 3D human corneal epithelium. Quality control of the MCTT HCE™ EIT model was performed by examining cell viability (MTT assay), histological data, and ET_{50} with 0.3% Triton-X100. Details on QC results were provided in the QC report (Appendix 8). For the transport of tissues, 24 tissues per a kit were placed on the top of agarose gel and delivered under refrigerated condition. Details of preparation, characterization, and original test method development of the MCTT HCE™ EIT were published (Jung *et al.*, 2011).

V. The Structure of Validation Management Team

This validation study for the MCTT HCE™ EIT was conducted with the support of the Korean Ministry of Food and Drug Safety (KMFDS). In this study, the validation management team (VMT) designated by Korean Center for the Validation of Alternative Method (KoCVAM) led the process of experiment design, selection of test materials, and review and approval of the test method. Fig. 1 shows the status of the VMT configuration and participating laboratories for this validation study.



[Figure 1] Organization of MCTT HCE™ EIT Validation Management Team

1. Validation Management Team (VMT)

The VMT was organized as shown in Fig.1 by KoCVAM. The VMT performed integrated management and scheduling of the entire validation process, selection and distribution of test materials, evaluation and statistical verification of test results, and drawing final conclusions. The VMT also discussed and approved the changes to the protocol, schedule and other deviations from the original project plan.

2. Trial Coordinator

By the agreement of the VMT committee members, Dr. Jeong, Tae Cheon (Yeungnam University) was appointed as the VMT Chairperson. The lead laboratory prepared a draft project plan, and VMT meetings were held with the participation of the VMT members and representatives from the lead laboratories. KoCVAM undertook the overall coordination work on this validation study and VMT operation.

3. Chemical Selection, Acquisition, Coding, and Distribution

The Chemical Selection Team in Fig.1 was responsible for selecting, listing, coding and supplying test materials. Dr. Ko, Kyung Yuk (KoCVAM) took charge of the overall test material management and Chemical Selection Team (CST) reporting.

4. Data Analysis (Biostatistician) Group

A biostatistics team was organized as shown in Figure 1, and was responsible for data analysis and statistical verification. Dr. Bae, SeungJin (Ewha Womans University) took charge of data analysis and statistical processing.

5. Lead Laboratories

Lead laboratories and participating laboratories were organized as shown in Fig. 1. Dr. Lim, Kyung-Min (Ewha Womans University), who was a test method developer, along with the AmorePacific R&D center, attended VMT meeting as the representative of the lead laboratories to present the introduction of the test method, test progress, and to respond to questions and comments from the VMT members. Dr. Lim was not involved in review of opinions on the validation study and did not participate in the selection or coding of test materials. Biosolution Co. (Dr. Lee, Su Hyon) joined the validation study as a co-lead laboratory and a participating laboratory in 2017.

6. Participating Laboratories

Four laboratories participated in the validation study. The College of Pharmacy, Ewha Womans University and AmorePacific R&D center were the first developers of the test method and became the co-lead laboratories in 2016. Biosolution Co. joined the validation study as a co-lead laboratory and a participating laboratory in 2017 since AmorePacific R&D center retired from the study due to an internal issue. Biotoxtech Co. and Korea Conformity Laboratories had acted as participating laboratories since 2016.

7. Sponsorship

VMT composition (final version):

Chair	Jeong, Tae Cheon (Yeungnam Univ., College of Pharmacy)
Scientific advisory members	Son, Youngsook (Kyunghee Univ., College of life Sciences) Chun, Young Jin (Chung-Ang Univ., College of Pharmacy) Bae, Ok-Nam (Hanyang Univ., College of Pharmacy) Kim, Ki-Suk (Korea Institute of Toxicology) Lee, Jeong lk (Konkuk Univ., College of Veterinary Medicine) Lee, Jong Kwon (KoCVAM)
Biostatistician	Bae, SeungJin (Ewha Womans Univ., College of Pharmacy)
Assistant administrator	Kim, Tae Sung (KoCVAM)
Chemical manager	Ko, Kyung Yuk (KoCVAM)
Lead laboratory	Lim, Kyung-Min (Ewha Womans Univ., College of Pharmacy)

8. Laboratories

Laboratories that performed the validation study are listed below. College of Pharmacy, Ewha Womans University served as the Lead laboratory from 2016 to 2017 and evaluated the transferability, BLR, WLR, and predictive capacity of the test method. Biosolution Co. served as a supportive role for the lead laboratory as a model developer and a participating laboratory. Korea Conformity Laboratories and Biotoxtech Co. joined as participating laboratories. The participating laboratories are geographically located apart from each other across South Korea and not affiliated mutually.

Lead Laboratory 1 (Study director: Lim, Kyung-Min)

Ewha Womans University, College of Pharmacy,
52 Ewhayeodae-gil, Seodaemun-gu, Seoul, Republic of Korea

Lead Laboratory 2 & Participating Laboratory 1 (Study director: Lee, Su Hyon)

Biosolution Co., Ltd.
Room 519, Seoul Techno Park, Gongneung-Ro 232, Nowon-Gu, Seoul, Republic of Korea

Participating Laboratory 2 (Study director: Kim, Jin Sik)

Korea Conformity Laboratories
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Participating Laboratory 3 (Study director: Jung, Mi Sook)

Biotoxtech Co., Ltd.
Safety Evaluation Team
686-2 Yangcheong-ri, Ochang-eup, Cheongwon-gu, Cheongju-si, Chungbuk, Republic of Korea

* Note: In addition to the laboratories listed above, two laboratories, AmorePacific R&D center and the Korea Testing & Research Institute (KTR), supported the validation trials for technical transfer and additional predictive tests, and these tests were conducted independently from the management and guidance of the VMT.

Trainer during Technical Transfer and Supplementary predictive test
(Study participant: Ms. Jang, Won-Hee)

AmorePacific Co.
314-1, Bora-ri, Yongin-si, Gyeonggi-do, Republic of Korea

Supplementary predictive test (Study participant: Ms. Yeo, Kyung Wook)

Korea Testing & Research Institute
12-63, Sandan-gil, Hwasun-eup, Hwasun-gun, Jeollanam-do, Republic of Korea

Table 1 below briefly shows the main roles of each laboratory that participated in this validation study.

[Table 1] Role of laboratories related to the validation study of MCTT HCE™ EIT

	Technical Transfer		**Main Study**			**Supplementary Study***
	Technical Transfer-Trainer	Technical Transfer-Trainee	Lead laboratory	Proficiency	WLR/BLR for PS	Additional predictive tests*

Ewha Womans University	O		O		O
Biosolution Co.		O	O	O	O	O
Korea Conformity Laboratories		O		O	O	O
Biotoxtech Co.		O		O	O	O
AmorePacific Co.	O					O
Korea Testing & Research Institute		O				O

*Additional predictive tests: in addition to the main predictive capacity test with those stated 30 reference chemicals, additional predictive tests were done with more reference chemicals other than 30 chemicals as to support the result of the predictive capacity of the test method, which was done independently from the current VMT.

VI. Study Design

1. Confirming similarity between MCTT HCE™ EIT and the EpiOcular™ EIT (OECD TG 492)

The MCTT HCE™ EIT is based on measuring the viability of cells that were exposed to test chemicals, and therefore, in principle, it is similar in principle and method to the EpiOcular™ EIT which is a VRM of OECD TG 492. A comparison of test components for the MCTT HCE™ EIT and the EpiOcular™ EIT is provided in Table 2.

[Table 2] Comparison of test components for the MCTT HCE™ EIT and the EpiOcular™ EIT

Test component (Required per performance standards for OECD TG 492)	MCTT HCE™ EIT		EpiOcular™ EIT	
Cell source (Relevant human-derived cells)	Primary human corneal epithelial cells		Primary human keratinocytes	
Pre-exposure (To select if necessary)	22 ± 2 h incubation		Pre-soak incubation	
Tissue replicates (Min. of 2 tissues)	2 tissues		2 tissues	
Application of test chemicals	Liquid	Solid	Liquid	Solid

Quantity (Uniformity)	40 µL (67 µL/cm^2)	40 mg (67 mg/cm^2)	50 µL (83 µL/cm^2)	50 mg (83 mg/cm^2)
Negative control (Determine as appropriate)	Dulbecco's phosphate buffered saline (D-PBS)		Ultrapure H_2O	
Positive control (Determine as appropriate)	2% SDS* in DDW** (or Methyl acetate, neat)		Methyl acetate	
Application period (Determine as appropriate)	10 ± 1 min	3 h ± 5 min	30 ± 2 min	6 h ± 15 min
Post-exposure soak (Optimize as appropriate)	None (Not required)	None (Not required)	12 ± 2 min	25 ± 2 min
Post-application period (Optimize as appropriate)	16 ± 1 h	16 ± 1 h	2 h ± 15 min	18 h ± 15 min
Cell viability measurement	WST-1 assay		MTT assay	
Cell viability threshold value (Determine as appropriate)	45%		60%	

* SDS: sodium dodecyl sulfate

** DDW: **distilled and de-ionized water**

The performance standards of OECD TG 492, GD 216 (OECD, 2015a) advises that a cell source, as well as appropriate parameters for the pre-exposure and application of test chemicals, are to be specified as necessary. Accordingly, parameters for each component of the MCTT HCE™ EIT have been optimized.

Cell viability is measured in the EpiOcular™ EIT using an MTT assay. In contrast, the MCTT HCE™ EIT uses WST-1 which produces a water soluble formazan dye upon reduction by a complex cellular mechanism that occurs primarily at the cell surface in NADPH dependent manner in viable cells. Unlike MTT which produces the insoluble MTT formazan crystal inside the cell and needs organic solvent extraction for optical density (OD) measurement, WST-1 makes a water soluble WST-1 formazan, which is spontaneously released into supernatant and therefore, does not need solvent extraction step (Ishiyama *et al.*, 1993; Tominaga *et al.*, 1999). The VMT considers these assays to be functionally equivalent since both are commonly used as tetrazolium-formazan reduction assays. **Also, by skipping organic extraction, the remaining tissues can be used for other assays that are incompatible with organic solvent extraction (e.g. RNA collections (Choi *et al.*, 2015), histology (Lee *et al.*, 2017) and lipid contents (Lee *et al.*, 2018)).** Color interference from the chemicals remaining in tissue after post-incubation (that would be most probably nonpolar) can also be reduced. Indeed, the color interference study, conducted by Biosolution Co., suggests that the color interference from colorful test chemicals is minimal and does not affect the ocular irritancy decisions made based on uncorrected viability, and the two other participating laboratories confirmed it (Appendix 12).

Thus, the VMT considered the MCTT HCE™ EIT to be a similar method to the RhCE EIT described in OECD TG 492, and this validation study for the MCTT HCE™ EIT was planned in accordance with the GD 216 performance standards (OECD, 2015a).

2. Test Chemicals

2.1 Chemical Selection

As listed in Table 3, the selected testing chemicals are the 30 reference chemicals listed in the OECD GD 216 performance standards (OECD, 2015a).

[Table 3] List of the 30 reference chemicals used in the MCTT HCE^{TM} EIT validation study

Irritant (I)				Non-Irritant (NI)			
No.	**Chemical name**	**CAS**	**UN GHS**	**No.**	**Chemical name**	**CAS**	**UN GHS**
1	(Ethylenediamine- propyl)-trimethoxysilane	1760-24-3	Cat 1	**16**	1-Ethyl-3- methylimidazolium ethylsulfate	342573-75-5	No Cat
2	Methylthioglycolate	2365-48-2	Cat 1	**17**	2-Ethoxyethyl methacrylate	2370-63-0	No Cat
3	Tetraethylene glycol diacrylate	17831-71-9	Cat 1	**18**	3-Phenoxybenzyl alcohol	13826-35-2	No Cat
4	1,2-Benzisothiazol-3(2H)-one	2634-33-5	Cat 1	**19**	4-(Methylthio)-benzaldehyde	3446-89-7	No Cat
5	2,5-Dimethyl-2,5-hexanediol	110-03-2	Cat 1	**20**	Dipropyl disulfide	629-19-6	No Cat
6	Disodium 2,2'-([1,1'-biphenyl]-4,4'-diyldivinylene)bis-(benzenesulfonate)	27344-41-8	Cat 1	**21**	Ethyl thioglycolate	623-51-8	No Cat
7	Sodium oxalate	62-76-0	Cat 1	**22**	Piperonyl butoxide	51-03-06	No Cat
8	2,4,11,13-Tetraazatetradecane-diimidamide, N,N''-bis(4-chlorophenyl)-3,12-diimino-, di-D-gluconate (20%, aqueous)	18472-51-0	Cat 2A	**23**	Polyethylene glycol (PEG-40) hydrogenated castor oil	61788-85-0	No Cat
9	gamma-Butyrolactone	96-48-0	Cat 2A	**24**	1-(4-Chlorophenyl)-3- (3,4-dichlorophenyl) urea	101-20-2	No Cat
10	1,5-Naphthalenediol	83-56-7	Cat 2A	**25**	2,2'-[[3-Methyl-4-[(4-nitrophenyl)azo]-phenyl]imino]bis-ethanol	3179-89-3	No Cat
11	Sodium benzoate	532-32-1	Cat 2A	**26**	2,2'-Methylene-bis-(6- (2H-benzotriazol-2-yl)-4-(1,1,3,3-tetramethylbutyl)-phenol)	103597-45-1	No Cat
12	2-Methyl-1-pentanol	105-30-6	Cat 2B	**27**	4,4'-Methylene bis-(2,6-di-tert-butylphenol)	118-82-1	No Cat
13	Diethyl toluamide	134-62-3	Cat 2B	**28**	Cellulose, 2-(2-hydroxy-3-(trimethylammonium) propoxy)ethyl ether chloride-91%	68610-92-4	No Cat
14	1,4-Dibutoxy benzene	104-36-9	Cat 2B	**29**	Potassium tetrafluoroborate	14075-53-7	No Cat
15	2,2-Dimethyl-3-methylenebicyclo [2.2.1] heptane	79-92-5	Cat 2B	**30**	Trisodium mono-(5-(1,2-dihydroxyethyl)-4-oxido-2-oxo-2,5- dihydro-furan-3-yl) phosphate	66170-10-3	No Cat

2.2 Coding and Distribution

KoCVAM, as the Chemical Selection Team (CST), selected test chemicals with the guidance of the VMT. The test chemicals were encoded (with different coding for each of three test run) and provided separately to each participating laboratory for each run by KoCVAM. Coding information is included as Appendix 2.

2.3 Test Phase

Depending on the circumstances and burden of each laboratory, and the limitation on the number of test substances that can be tested at a time, thirty reference chemicals were divided and tested over two or three phases for each test run. Testing procedures were the same for all phases. Table 4 below is a flowchart showing an overview on the different study phases of each laboratory for each test run.

[Table 4] Overview on the different study phases of each laboratory

Lab 1*					**Lab 2****				**Lab 3*****		
	Run 1	**Run 2**	**Run 3**		**Run 1**	**Run 2**	**Run 3**		**Run 1**	**Run 2**	**Run 3**
Phase 1	15 Ref. chem.	15 Ref. chem.	15 Ref. chem.	**Phase 1**	10 Ref. chem.	10 Ref. chem.	10 Ref. chem.	**Phase 1**	15 Ref. chem.	10 Ref. chem.	10 Ref. chem.
Phase 2	15 Ref. chem.	15 Ref. chem.	15 Ref. chem.	**Phase 2**	10 Ref. chem.	10 Ref. chem.	10 Ref. chem.	**Phase 2**	15 Ref. chem.	10 Ref. chem.	10 Ref. chem.
Phase 3	ND	ND	ND	**Phase 3**	10 Ref. chem.	10 Ref. chem.	10 Ref. chem.	**Phase 3**	ND	10 Ref. chem.	10 Ref. chem.

* Lab 1; Biosolution Co., **Lab 2; Korea Conformity Laboratories, ***Lab 3; Biotoxtech Co. ND, Not done

3. Study Quality Criteria

The study was conducted in compliance with Good Laboratory Practice (GLP) principles. Two laboratories (Biotoxtech and Korea Conformity Laboratories) are the GLP-accredited contract research organizations. Biosolution Co. conducted the tests in the GLP spirit. Quality Assurance (QA) sheet was checked by KoCVAM at every run to ensure the quality of the study.

4. Defined Reliability and Accuracy Value

4.1 Transferability of the Test Method

Two participating labs (Korea Conformity Laboratories and Biosolution Co.) were trained for the test method by AmorePacific R&D center, an original method developer, based on the draft protocol 1.4. Transferability was checked for eight chemicals by Korea Conformity Laboratories and five chemicals by Biosolution Co. as shown below in Table 5.

[Table 5] Results of transferability tests in two participating labs, Korea Conformity Laboratories and Biosolution Co.

	Chemical	CAS No.	State	GHS	VRM	MCTT HCE™ Viability (%)	Decision	Pass/Fail
Korea Conformity Laboratories	Sodium oxalate	62-76-0	Solid	Cat 1	I	**9.0 ± 0.5**	**I**	**Pass**
	2,4,11,13-Tetraazatetradecane-diimidamide, N,N''-bis(4-chlorophenyl)-3,12-diimino-, di-D-gluconate	18472-51-0	Liquid	Cat 2A	I	**17.9 ± 4.4**	**I**	**Pass**
	Sodium benzoate	532-32-1	Solid	Cat 2A	I	**17.6 ± 0.2**	**I**	**Pass**
	2-Methyl-1-pentanol	105-30-6	Liquid	Cat 2B	I	**22.3 ± 0.5**	**I**	**Pass**
	1-Ethyl-3-methylimidazolium ethylsulphate	342573-75-5	Liquid	No Cat.	NI	**89.1 ± 4.3**	**NI**	**Pass**
	Polyethylene glycol (PEG-40) hydrogenated castor oil	61788-85-0	Viscous	No Cat.	NI	**104.7 ± 4.8**	**NI**	**Pass**
	1-(4-Chlorophenyl)-3-(3,4-dichlorophenyl) urea	101-20-2	Solid	No Cat.	NI	**130.6 ± 0.2**	**NI**	**Pass**
	2,2'-Methylene-bis-(6-(2H-benzotriazol-2-yl)-4-(1,1,3,3-tetramethylbutyl)-phenol)	103597-45-1	Solid	No Cat.	NI	**128.2 ± 5.2**	**NI**	**Pass**
Biosolution Co.	Promethazine hydrochloride	58-33-3	Solid	Cat 1	I	**1.1 ± 0.0**	**I**	**Pass**
	Methylthioglycolate	2365-48-2	Liquid	Cat 1	I	**23.5 ± 0.5**	**I**	**Pass**
	2,5-Dimethyl-2,5-hexanediol	110-03-2	Solid	Cat 1	I	**1.7 ± 0.0**	**I**	**Pass**
	Potassium tetrafluoroborate	14075-53-7	Solid	No Cat	NI	**110.3 ± 11.2**	**NI**	**Pass**

Piperonyl butoxide	1951-03-06	Liquid	No Cat	NI	**122.8 ± 2.3**	**NI**	**Pass**

* Detailed information on the transferability is available in Appendix 4. Viability values are means ± 1/2 difference of duplicate wells or means ± SD of ≥ 3 wells.
(Note: Biosoluiton Co. conducted transferability test with total of 4 wells.)

4.2 Within-Laboratory Reproducibility (WLR)

The OECD GD 216 performance standards (OECD, 2015a) advises that the RhCE EIT similar to TG 492 shall meet the within-laboratory reproducibility of equal to or higher than 90%. A total of three runs were conducted for each of the 30 reference chemicals by each participating laboratory. The ratio of concordance of decisions within three runs was calculated into percent reproducibility. The results of WLR are presented in section VIII.

4.3 Between-Laboratory Reproducibility (BLR)

The OECD GD 216 performance standards (OECD, 2015a) advises that the RhCE EIT similar to TG 492 shall meet the between-laboratory reproducibility of equal to or higher than 85%. A total of three runs were conducted for each of the 30 reference chemicals by each participating laboratory. The ratio of concordance of decisions made from the average viability of each laboratory was calculated into percent reproducibility. The results of BLR are presented in section VIII.

4.4 Predictive Capacity

The OECD GD 216 performance standards (OECD, 2015a) describes that the acceptable criteria for predictive capacity is equal or better than 90% sensitivity, 60% specificity and 75% accuracy.

To evaluate the predictive capacity of the EIT using MCTT HCE™ models, the 30 reference chemicals in the OECD GD 216 performance standards were tested three times by each participating laboratory. Nine runs were done for each chemical (three runs by three laboratories), which results in 270 runs in total. The predictive capacity was calculated in three different ways: 1) 270 runs were estimated independently, 2) based on the decision of 30 chemicals from the average cell viability from each laboratory, or 3) based on the decision of 30 chemicals from majority votes~~, respectively~~.

Additionally, the data for 141 reference chemicals (130 chemicals tested to evaluate predictive capacity of MCTT HCE™ EIT, which were selected from the ECETOC database (Bagley *et al.*, 1999), nine reference chemicals in OECD GD216 non-overlapped with 130 chemicals, and new PS 2 chemicals added in the revised OECD GD216 in 2017) with known *in vivo* irritancy, composed of 87 liquids and 54 solids (80 irritants and 61 non-irritants) were evaluated to assess the predictive capacity of MCTT HCE™ EIT. Of 130 chemicals previously tested, 81 liquids were tested according to protocol 1.2 or 1.4 which is compatible to protocol 1.6 but 49 solids were not compatible, therefore, re-tested based on protocol 1.6. The agreement of binary decisions of irritant or non-irritant with *in vivo* classification (Category 1 or 2A/2B vs. No category) was assessed. Predictive capacity was presented as sensitivity, specificity, and

accuracy. For the chemicals tested more than once, an arithmetic mean value was obtained and used for the decision. The results of the predictive capacity are presented in section VIII. Other approaches like separate cutoffs for liquid and solid, and weighted assessment were also assessed and presented in section IX.

5. Data Collection, Handling, and Analysis

The lead laboratory, Ewha Womans University collected and tabulated data sheets from participating laboratories in coded status at the end of each run and provided them to KoCVAM. Then, KoCVAM provided the coding information for test chemicals after checking the validity of data.

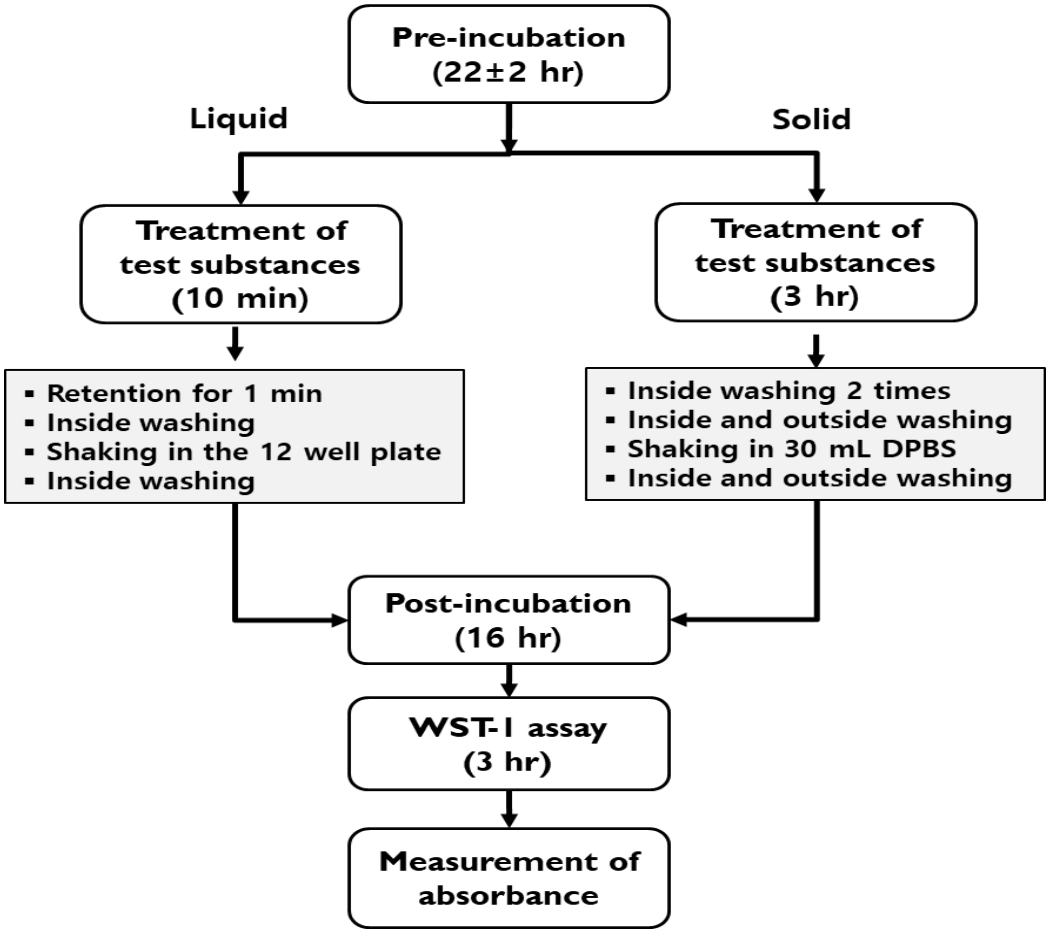
A final data summary was analyzed by an independent biostatistician (Dr. Bae, SeungJin). The results were reported to, and approved by the VMT.

VII. Protocol

A protocol for the MCTT HCE™ EIT was developed by the AmorePacific R&D center and enacted as ver. 1.0 on 15th June, 2014. After six times of revisions, the protocol was finalized as 1.6. Detailed protocol and revision history were included in Appendix 3. The main validation data (i.e., WLR, BLR and predictive capacity based on 30 reference chemicals) was conducted according to protocol ver. 1.5 or 1.6 (which are essentially identical except for the inclusion of methyl acetate as an additional PC).

1. Protocol for the MCTT HCE™ EIT

MCTT HCE™ models, which passed the quality control (OD values of negative control: 0.8 ~ 1.2, ET_{50}: 17.6 ~ 41.0 min and histological examination) of the manufacturer, is delivered to the testing labs in a 24-well format on agarose gel in refrigerated condition. Upon receipt of the shipment, culture medium is pre-warmed in a 37°C thermostat for 30 min. As preparation for the pre-incubation step, 900 μL of the pre-warmed medium is added to each well of a 6-well plate using micropipette and the HCE model insert is carefully transferred to the wells using a forceps. Then the well-plate is pre-incubated at 37°C, 5% CO_2 for 22±2 hr. To conduct the experiment, 40 μL of liquid substance or solution, or 40 mg of solid substance is topically applied to the upper epithelial surface of the model insert (0.6 cm^2) after the pre-incubation. Then the tissue is incubated again (37°C, 5% CO_2 condition) for 10 ± 1 min or 3 hrs ± 5 min depending on the physical state of the test substance (Fig. 2). Then the tissue is washed to remove the test substances and further incubated for 16 ± 1 hr. The resulting tissue viability is evaluated with WST-1 assay.

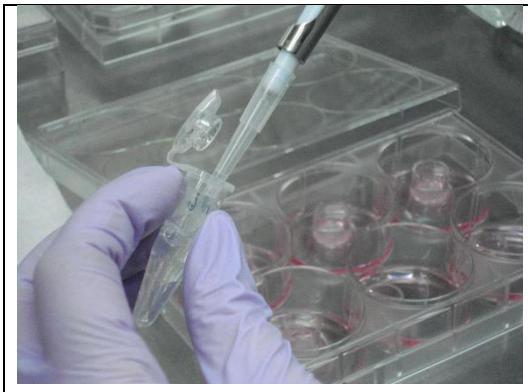


[Figure 2] Overview of MCTT HCE^{TM} eye irritation test procedure

1.1 MCTT HCE^{TM} EIT Protocol for Liquid Chemicals

Application of the chemical

Liquid test chemicals should be applied under the chemical hood since there are many chemicals that are volatile and odorous. Take 40 μL of the test material with a micropipette and slowly drop it atop and at the center of the cornea model (Fig. 3). Rotate the insert uniformly to apply to the whole surface. When application of the test chemical in 6-well units is done, put it in a 5% CO_2 cell incubator at 37°C and incubate it for 10 ± 1 min.




[Figure 3] Application of liquids; Pipette 40 μL of liquid test material (left), dispense directly atop the tissue and spread gently (right).

Washing

Washing process of the 6-wells should be done within 20 min. DPBS for washing should be kept warm in a 37°C thermostat before use. During washing, place 2-3 cm of distance between the tip of the pipette aid and the tissue, and flow DPBS along the well-wall. Remaining DPBS in-between each washing step is left until the final washing and removed at once.

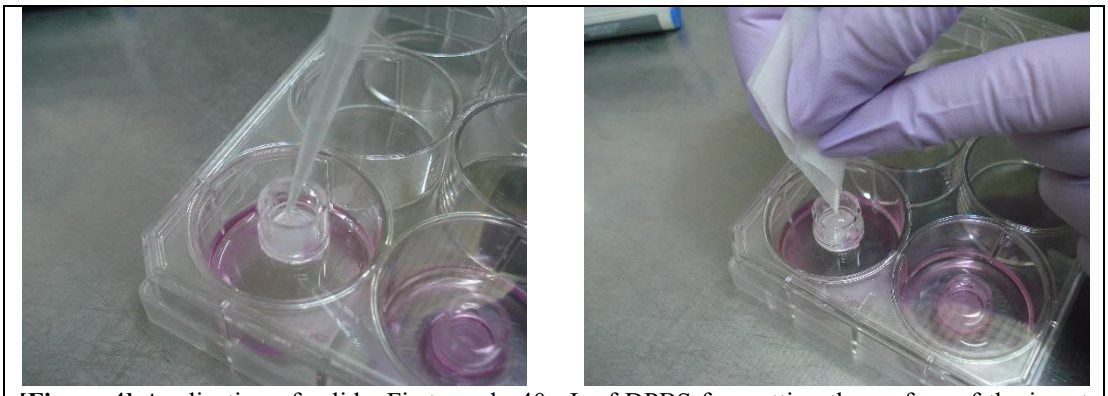
- Detailed procedures are as below:

1) After 10 ± 1 min of incubation, take out the plate from the incubator. Add 4 mL of DPBS, using pipette aid, into the insert to overflow the material inside, and leave for about 1 min. Then, flip over the insert using forceps to remove the material inside the insert. The DPBS washing interval between the wells should be the same as the application interval. For example, if the chemical is applied at 30 sec. intervals, washing should also be performed at 30 sec. intervals.
2) Take 10 mL of DPBS using pipette aid to wash the insert. Hold the insert with forceps and apply DPBS to the insert to remove all material inside. 10 mL of DPBS is applied in approximately 10 sec. increments (1 mL/sec.). Washing procedure of this test using DPBS should be maintained at this rate.
3) Place those 6 inserts in each well of a 12-well plate. At 10 sec intervals, add 4 mL of DPBS to the insert in each well to overflow the material inside, and leave for about 1 min. Then grab the insert with forceps and gently shake it 5 times in DPBS to remove debris and place the inserts on the cover of the 12-well plate.
4) Hold the insert with forceps and rinse inside and outside of the insert thoroughly with 10 mL of DPBS using pipette aid.
5) Finally, remove residual DPBS inside the insert using a micropipette and by absorbing with the sterilized gauze, remove DPBS on the outside of the insert as well.

1.2 MCTT HCE^{TM} EIT Protocol for Solid Chemicals

Application of the chemical

Start the procedure by taking the cornea model out of the 6 wells and place it on a cover of the well. First of all, wet the surface of the cornea model with 40 μL of DPBS using a micropipette, then apply 40 mg of the test chemical atop and at the center of the cornea model. Then, gently shake the insert to allow the chemical to spread evenly over the entire surface (Fig. 4). Once application of the test chemical is completed in 6 well units, put it in a 5% CO_2 cell incubator at 37℃ for 3 hr ± 5 min.



[Figure 4] Application of solids; First, apply 40 μL of DPBS for wetting the surface of the insert (left), and then apply the weighed test chemical to the insert.

Washing

Washing process of the 6-wells should be done within 20 min. DPBS for washing should be kept warm in a 37°C thermostat before use. During washing, place 2-3 cm of distance between the tip of the pipette aid and the tissue, and flow DPBS along the well-wall. Remaining DPBS in-between each washing step is left until the final washing and removed at once.

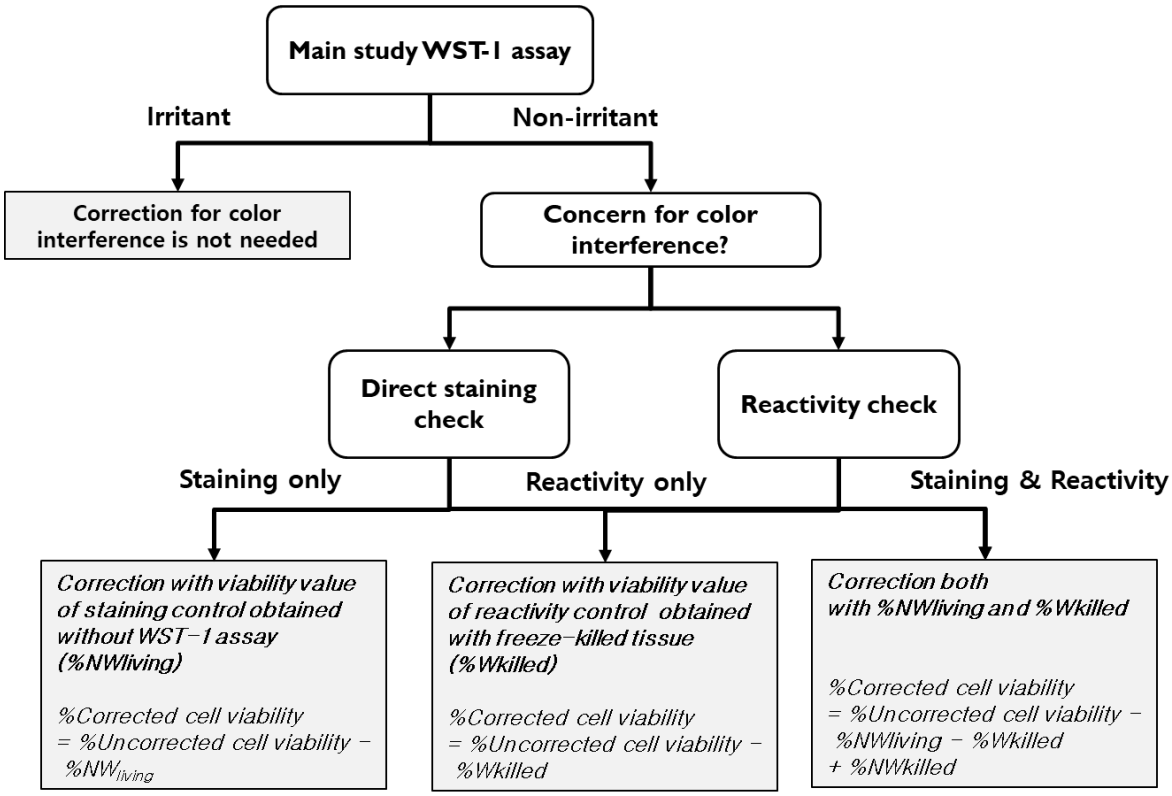
- Detailed procedures are as below:

1) After the 3 hr ± 5 min of incubation procedure, take the plate out of the incubator. Add 10 mL of DPBS using pipette aid to the insert for washing. Hold the insert with forceps and apply DPBS to the insert to remove materials inside. The DPBS washing interval between the wells should be the same as the application interval. If most of the solid material is not removed during this process, gently wipe out only the solid material with a sterile swab. Be careful not to scratch or damage the surface of the cornea model.
2) Repeat the steps of washing.
3) Pick up the insert with forceps and rinse with 10 mL of DPBS using an auto pipette. Turn the insert back and forth when rinsing with DPBS so that the both sides of the insert are rinsed thoroughly.
4) To remove the debris, hold the insert with forceps and gently shake the insert more than 5 times in a 50 mL beaker containing 30 mL of DPBS. Each time the insert gets rinsed in DPBS, take the insert out of the beaker and turned upside down to remove the liquid inside the insert. If remaining solid is still observed by visual inspection, wash it sufficiently at this stage. If the entire solid is not removed even after washing up to 10 times, do not wash it any more to prevent damage of the cornea. Beakers should be used separately for each material.
5) Repeat step 3).

6) Finally, remove residual DPBS inside the insert using a micropipette and by absorbing with the sterilized gauze, remove the DPBS on the outside of the insert as well.

1.3 Detecting and Correcting Interference from Colorful Chemicals and/or test chemicals with WST-1 reactivity

In order to prevent the possible interference with the WST-1 endpoint from colorful chemicals, 'direct staining by test materials' may be checked. Though, when a test substance is determined to be an irritant, the correction is not necessary since correction always over-predict the classification of ocular irritancy by subtracting the cell viability. Moreover, WST-1 assay does not require organic extraction as in MTT assay, which can reduce the color interference from test chemicals significantly. Accordingly, the color interference corrections are reserved as optional for the test chemicals with significant concerns and at the same time, determined as non-irritants based on viability. Overall scheme for correcting color interference is as follows,



[Figure 5] Overall flow-chart for correcting color interference

Checking for direct staining

Colorful test substance may interfere with OD measurement of WST-1 formazan by directly staining the tissue.

Add 1 mL of deionized water and 40 μL (liquid) or 40 mg (solid) of the test chemical to each well of a 24-well plate and incubate the mixture in a cell incubator (37 ± 1°C, 5 ± 1 % CO_2, 95% RH) for 60

min. Take 200 µL of the reacted solution in duplicate and transfer to a 96-well plate for OD measurement. If an OD value measured at 450 nm is more than 0.1, then correction as described below may be necessary.

Checking for WST-1 reactivity

Test substance with reducing potential may reduce WST-1 directly and form formazan by itself.

Add 40 µL (liquid) or 40 mg (solid) of the test substance into 1 mL of diluted working WST-1 solution (1:25). Incubate the mixture in the incubator (37 ± 1°C, 5 ± 1 % CO_2, 95% RH) for 60 min. At the end of the exposure time, shake the mixture and evaluate the presence of formazan. If the solution changes color significantly, the test substance is presumed to have the potential to reduce WST-1. Correction of the cell viability may be necessary as explained below.

Correction of cell viability for the interference of WST-1 assay

1) When the test substance only has color interference (direct staining);

- The test substance may color-stain the tissue. Conduct the test according to the main protocol but use DPBS instead of WST-1 solution (NSC_{living} [Non-specific colour in living tissues control]). This shall be done in duplicate and used as an additional negative control for the test substance in calculating cell viability based on the following equation,

$$True\ tissue\ viability = [\%Viability_{test}] - [\%NSC_{living}]$$

2) When the test substance exhibits no color-staining but direct WST-1 reduction;

- Conduct the test according to the main protocol but use a freeze-killed tissue (NSWST-1 [Non-specific WST-1 reduction control]) instead of a viable tissue. The freeze-killed tissue has no metabolic activity but capable of absorbing and binding the test substance. Upon receipt of the tissue, the tissue is frozen in 24-well plate at -80°C for 48 hr. Then the tissue is transferred into 6-well plate containing fresh medium for 10 min to stabilize. The resulting cell viability is used to correct cell viability as follows,

$$True\ tissue\ viability = [\%Viability_{test}] - [\%NSWST\text{-}1]$$

  This functional check is not done at every test run but can be done at once for each test substance with concern in duplicate.

3) When the test substance has both color interference and direct WST-1 reduction;

- In this case, the correction shall be done for both color interference and WST-1 reduction as described in 1.3 1) and 2) but the color interference will be subtracted twice. Therefore, another functional check with freeze-killed tissue is conducted with DPBS instead of WST-1 solution (NSC_{killed} [Non-specific colour in killed tissues control]). Freeze-killed tissue is prepared as described above and the test is conducted in duplicate once for each test substance. Final cell viability is calculated as follows,

$$True\ tissue\ viability = [\%Viability_{test}] - [\%NSWST\text{-}1] - [\%NSC_{living}] + [\%NSC_{killed}]$$

2. Prediction Model for the MCTT HCE^{TM} EIT

According to the EU and the GHS classification, an irritant is predicted if the mean relative tissue viability of minimum of two individual tissues exposed to the test chemical is reduced to 45% or below of the mean viability of the negative controls. The test chemical was defined as a non-irritant if the tissue viability was higher than 45%. Otherwise, it was determined as an irritant (Table 6).

[Table 6] Prediction model for the MCTT HCE^{TM} EIT

Prediction model	Classification
Mean tissue viability is ≤ 45%	Irritant (I)
Mean tissue viability is > 45%	Non-Irritant (NI)

2.1 Optimized Prediction Model Cut-offs for Liquids and Solids

Although the original cutoff value is 45% when considering both liquid and solid chemicals together, it was suggested that the application of separate cutoff values for liquids and solids may improve the predictive capacity of the MCTT HCE^{TM} EIT. To confirm the validity of the idea, ROC curves were redrawn separately for liquids and solids, and the WLR, BLR and predictive capacity were re-assessed with separate cutoffs as well.

3. Acceptance Criteria

3.1 Negative Control

The negative control chemical is DPBS, and the OD value of the negative control chemical should be 1.6 or more and 3.0 or less.

$$1.6 \leq \text{Mean OD measured value} \leq 3.0$$

3.2 Positive Control

The cell viability of the positive control chemical is used as a quality control index. The positive control material should be 2% SDS or methyl acetate and the average cell viability of the positive control should be 35% or less.

$$\text{Mean tissue viability of positive control} \leq 35\%$$

3.3 Criteria for a Re-test

A test is regarded as acceptable as long as results do not fall into the criteria for a re-test shown below.

Criteria for a re-test

1) If a WST-1 absorbance value of the negative control chemical is less than 1.6 or exceeds 3.0.
2) If a cell viability value of the positive control chemical is more than 35%.

3) In negative control chemical, positive control chemical, test chemical: When the difference of their cell viability value of treated tissue in replications exceeds 20%.

4) If the average cell viability of the chemical-treated-well is ≥ 40% and ≤ 50%.

(Note: If the re-test gives borderline value again, the concordance of the decision shall be considered. If the original and re-test are concordant in decision, concordant decision is taken and additional re-test is not necessary. If the decision diverges, then the third test shall be conducted and majority vote approach shall be taken for final decision)

3.4 Standard Deviation or Difference between Duplicate Wells

Since eye irritation potential is predicted from the mean viability of tissues, the variability of tissue replicates must be kept at an acceptably low level.

When three replicate tissues are used, the standard deviation (SD) of tissue viability of three identically treated replicates for the negative control, the positive control, and test chemicals shall be less than 18%, or when duplicate tissues are used, the difference between the viabilities of duplicate tissues shall not exceed 20%. Current study was done employing duplicate tissues per a test chemical, negative control or positive control.

VIII. Results

1. QC of the Tissue Models

To ensure the quality of a manufactured MCTT HCE™ model, each batch was tested for the cell viability of the negative control (with respect to OD with MTT assay) and ET_{50} (an exposure time estimated to reduce the cell viability to 50% with a non-ionic detergent, 0.3% Triton-X 100 as measured with MTT assay after the exposure to 0.3% Triton-X 100 for 0, 15, 30, 45, and 60 min. More specifically, Viability-Exposure time relationship graph for 4 points of time and viability is drawn on semi-log scale and ET_{50} is calculated based on regression. Details are described in Appendix 8). In addition, histological examination was conducted. The quality control tests were done with the tissue stored in a condition similar to the one actually delivered to the test site (at 5 °C for at least 3 hrs. and pre-incubated overnight). QC tests have been done for all batches used in the validation study as reported in Appendix 8.

At testing sites, the negative control was DPBS, and the OD value of the negative control should be ≥ 1.6 and ≤ 3.0. The positive control should be 2% SDS or methyl acetate (neat) and the average cell viability of the positive control should be 35% or less.

2. Quality Assurance

Assays and quality assurance were carried out in the spirit of GLP, although not all participating laboratories were GLP certified. The participating laboratories conducted the experiments according to Protocol 1.6. Essential steps of the test were checked during the conduct of the test. All raw data and data

sheets were reviewed at each laboratory and then checked for errors and omissions by the VMT data analysis group. The results accurately reflected the raw data (Appendix 6).

3. Negative Control

Table 7 shows absorbance values for the negative control for three runs by three laboratories. It includes re-test runs which were required by the occurrence of invalid runs according to re-test criteria (mostly, they were due to borderline results). All data satisfied the acceptance criteria for the negative control, 1.6 ≤ mean OD ≤ 3.0 (Actual values, 1.87-2.93).

[Table 7] Absorbance values for the negative control for 3 runs by three laboratories

	Lab 1			**Lab 2**			**Lab 3**		
	1	2	3	1	2	3	1	2	3
Phase 1	2.93	2.81	2.77	2.54	2.78	2.35	2.48	2.77	2.91
Phase 2	2.80	2.67	2.85	2.44	1.93	2.79	2.60	2.86	2.76
Phase 3	-	-	-	2.62	1.91	2.77	-	2.76	2.89
Re-test*	2.36	2.46	2.56	-	-	-	1.87	2.88	2.89

Lab 1; Biosolution Co., Lab 2; Korea Conformity Laboratories, Lab 3; Biotoxtech Co.
*Re-tests were done due to borderline results meeting the re-test criteria #4 (16 cases) or criteria #3 (3 cases)

4. Positive Control

Table 8 shows absorbance values and cell viabilities for the positive control (SDS 2% or methyl acetate) for 3 runs by three laboratories. All data satisfied the acceptance criteria for the positive control, and mean cell viability was ≤ 35%.

[Table 8] Cell viabilities (%) for the positive control for 3 runs by three laboratories

	Lab 1			**Lab 2**			**Lab 3**		
	1	2	3	1	2	3	1	2	3
Phase 1	4.85^{+}	16.85	6.25^{+}	11.27	7.87	6.70	14.93	5.85	7.94
Phase 2	25.80	33.15	9.30^{+}	15.67	4.77	2.73	10.73	5.83	10.36
Phase 3	-	-	-	9.35	7.55	5.49	-	7.33	9.39
Re-test****	20.85	5.3	9.10^{+}	-	-	-	20.31	10.83	12.70

Lab 1; Biosolution Co., Lab 2; Korea Conformity Laboratories, Lab 3; Biotoxtech Co.
*Re-tests were done due to borderline results meeting the re-test criteria #4 (16 cases) or criteria #3 (3 cases),
+, Methyl acetate as PC.

5. Reliability

5.1 Within-Laboratory Reproducibility (WLR)

WLR at the three laboratories (Lab 1, Lab 2 and Lab 3) was evaluated (Protocol 1.6) for 30 reference chemicals. According to the performance standards, WLR was evaluated by estimating concordance in

classification (No category or “No prediction made”) for three runs for each laboratory. As shown in Table 9, WLRs were 90 to 100%, which met the PS criteria of WLR, ≥ 90%.

[Table 9] Within-laboratory reproducibility for 30 reference chemicals

		Concordance (%)	Pass/ failure for WLR
Lab 1	Run 1~3	100%	Pass
Lab 2	Run 1~3	90.0%	Pass
Lab 3	Run 1~3	90.0%	Pass

Lab 1; Biosolution Co., Lab 2; Korea Conformity Laboratories, Lab 3; Biotoxtech Co.

5.2 Between-Laboratory Reproducibility (BLR)

BLR among three laboratories that passed WLR criteria was evaluated for the concordance in classification of 30 reference chemicals listed in the performance standards.

According to the OECD GD 216 PS, BLR was evaluated for the 30 reference chemicals by estimating concordance in classification (No category or “No prediction made”) made with the averaged viability value of each laboratory. A criterion for BLR stated in the PS is ≥ 85%. As shown in Table 10, BLR was 90%, which met PS criteria of BLR, ≥ 85%.

[Table 10] Between-laboratory reproducibility for 30 reference chemicals

No	Chemical	CAS No.	Physical state	UN GHS	Final prediction (1-3)			Concordance
					Lab 1	Lab 2	Lab 3	
1	(Ethylenediamine-propyl)-trimethoxysilane	1760-24-3	liquid	Cat1	TP	TP	TP	Y
2	Methylthioglycolate	2365-48-2	liquid	Cat1	TP	TP	TP	Y
3	Tetraethylene glycol diacrylate	17831-71-9	liquid	Cat1	TP	TP	TP	Y
4	1,2-Benzisothiazol-3(2H)-one	2634-33-5	solid	Cat1	TP	TP	TP	Y
5	2,5-Dimethyl-2,5-hexanediol	110-03-2	solid	Cat1	TP	TP	TP	Y
6	Disodium 2,2'-([1,1'-biphenyl]-4,4'-diyldivinylene)bis-(benzenesulphonate)	27344-41-8	solid	Cat1	TP	TP	TP	Y
7	Sodium oxalate	62-76-0	solid	Cat1	TP	TP	TP	Y
8	2,4,11,13-Tetraazatetradecane-diimidamide,N,N''-bis(4-chlorophenyl)-3,12-diimino-,di-D-gluconate (20%,aqueous)	18472-51-0	liquid	Cat2A	TP	TP	TP	Y
9	gamma-Butyrolactone	96-48-0	liquid	Cat2A	TP	TP	TP	Y
10	1,5-Naphthalenediol	83-56-7	solid	Cat2A	TP	TP	FN	N
11	Sodium benzoate	532-32-1	solid	Cat2A	TP	TP	TP	Y
12	2-Methyl-1-pentanol	105-30-6	liquid	Cat2B	TP	TP	TP	Y
13	Diethyl toluamide	134-62-3	liquid	Cat2B	TP	TP	TP	Y
14	1,4-Dibutoxy benzene	104-36-9	solid	Cat2B	FN	FN	FN	Y
15	2,2-Dimethyl-3- methylenebicyclo [2.2.1] heptane	79-92-5	solid	Cat2B	TP	TP	TP	Y
16	1-Ethyl-3- methylimidazolium ethylsulphate	342573-75-5	liquid	NC	TN	TN	TN	Y
17	2-Ethoxyethyl methacrylate	2370-63-0	liquid	NC	FP	FP	FP	Y
18	3-Phenoxybenzyl alcohol	13826-35-2	liquid	NC	FP	FP	FP	Y
19	4-(Methylthio)-benzaldehyde	3446-89-7	liquid	NC	FP	FP	FP	Y

20	Dipropyl disulphide	629-19-6	liquid	NC	TN	FP	TN	N
21	Ethyl thioglycolate	623-51-8	liquid	NC	FP	FP	FP	Y
22	Piperonyl butoxide	51-03-06	liquid	NC	TN	TN	TN	Y
23	Polyethyleneglycol(PEG-40) hydrogenated castor oil	61788-85-0	viscous	NC	TN	TN	TN	Y
24	1-(4-Chlorophenyl)-3- (3,4-dichlorophenyl) urea	101-20-2	solid	NC	TN	TN	TN	Y
25	2,2'-[[3-Methyl-4-[(4-nitrophenyl)azo]-phenyl] imino]bis-ethanol	3179-89-3	solid	NC	FP	TN	TN	N
26	2,2'-Methylene-bis-(6- (2H-benzotriazol-2-yl)-4-(1,1,3,3-tetramethylbutyl)-phenol)	103597-45-1	solid	NC	TN	TN	TN	Y
27	4,4'-Methylene bis-(2,6-di-tert-butylphenol)	118-82-1	solid	NC	TN	TN	TN	Y
28	Cellulose, 2-(2-hydroxy-3-(trimethylammonium)propoxy)ethyl ether chloride (91%)	68610-92-4	solid	NC	TN	TN	TN	Y
29	Potassium tetrafluoroborate	14075-53-7	solid	NC	TN	TN	TN	Y
30	Trisodium mono-(5-(1,2-dihydroxyethyl)-4-oxido-2-oxo-2,5-dihydro-furan-3-yl) phosphate	66170-10-3	solid	NC	FP	FP	FP	Y
					Between-Laboratory Reproducibility			90.0% (27/30)

TP; True positive; TN; True negative; FP; False positive; FN; False negative

Lab 1; Biosolution Co., Lab 2; Korea Conformity Laboratories, Lab 3; Biotoxtech Co.

6. Predictive Capacity

6.1 Predictive Capacity for 30 Reference Chemicals

30 reference chemicals from the performance standards of OECD GD 216 (OECD, 2015a) were evaluated by three laboratories in three repetitions, which led to a total of 270 determinations as shown in the table below (Table 11). The test results were uncorrected for color interferences since correction scheme as shown in VII.1.3 led to identical results when tested by Biosolution Co. (Lab 1) (Appendix 12).

[Table 11] Individual test results for 30 reference chemicals conducted three times by three participating laboratories

No	Chemical	CAS No.	Physical state	UN GHS	Lab 1			Lab 2			Lab 3		
					1	2	3	1	2	3	1	2	3
1	(Ethylenediamine-propyl)-trimethoxysilane	1760-24-3	liquid	Cat1	31.80	10.85	32.45	24.68	17.02	29.95	28.09	16.17	39.45
2	Methylthioglycolate	2365-48-2	liquid	Cat1	21.30	32.10	22.35	13.08	11.89	22.85	24.39	17.27	24.42
3	Tetraethylene glycol diacrylate	17831-71-9	liquid	Cat1	2.30	3.10	2.65	2.41	1.78	2.17	3.57	3.50	3.15

4	1,2-Benzisothiazol-3(2H)-one	2634-33-5	solid	Cat1	4.00	0.65	0.95	0.72	0.63	0.43	0.61	0.78	0.78
5	2,5-Dimethyl-2,5-hexanediol	110-03-2	solid	Cat1	0.45	0.55	0.55	0.51	0.67	0.29	5.83	0.49	1.15
6	Disodium 2,2'-([1,1'-biphenyl]-4,4'-diyldivinylene)bis-(benzenesulphonate)	27344-41-8	solid	Cat1	16.00	10.20	30.75	6.44	8.65	2.69	3.23	12.52	25.58
7	Sodium oxalate	62-76-0	solid	Cat1	30.50	36.90	24.35	38.09	18.81	32.17	21.87	46.96	33.31
8	2,4,11,13-Tetraazatetradecane-diimidamide,N,N''-bis(4-chlorophenyl)-3,12-diimino-,di-D-gluconate (20%,aqueous)	18472-51-0	liquid	Cat2A	1.90	1.85	1.75	1.95	1.24	11.78	2.33	2.05	1.58
9	gamma-Butyrolactone	96-48-0	liquid	Cat2A	3.10	2.40	3.55	5.52	3.29	3.40	2.46	1.94	5.17
10	1,5-Naphthalenediol	83-56-7	solid	Cat2A	39.40	38.05	35.85	62.97	32.10	25.18	50.87	56.59	80.43
11	Sodium benzoate	532-32-1	solid	Cat2A	0.85	1.70	0.70	0.85	1.50	0.52	0.32	0.85	4.04
12	2-Methyl-1-pentanol	105-30-6	liquid	Cat2B	2.55	1.65	9.15	2.40	0.88	3.72	2.30	3.32	2.31
13	Diethyl toluamide	134-62-3	liquid	Cat2B	0.85	1.30	4.85	2.75	5.20	1.11	4.33	2.19	8.66
14	1,4-Dibutoxy benzene	104-36-9	solid	Cat2B	97.60	73.25	96.65	103.18	102.98	105.63	104.83	99.46	102.37
15	2,2-Dimethyl-3-methylenebicyclo [2.2.1] heptane	79-92-5	solid	Cat2B	31.80	9.90	27.05	23.26	15.67	6.48	4.39	30.01	35.88
16	1-Ethyl-3-methylimidazolium ethylsulphate	342573-75-5	liquid	NC	55.15	54.45	60.65	68.21	59.46	60.01	24.49	56.88	65.37
17	2-Ethoxyethyl methacrylate	2370-63-0	liquid	NC	0.90	1.20	1.85	1.11	0.77	7.40	0.87	1.10	1.44
18	3-Phenoxybenzyl alcohol	13826-35-2	liquid	NC	9.80	5.50	25.00	16.10	26.40	7.41	0.73	20.89	15.57
19	4-(Methylthio)-benzaldehyde	3446-89-7	liquid	NC	10.50	27.30	22.55	9.95	5.76	68.59	9.12	7.25	31.95
20	Dipropyl disulphide	629-19-6	liquid	NC	46.45	51.60	55.20	69.70	8.62	14.74	52.26	77.54	55.68
21	Ethyl thioglycolate	623-51-8	liquid	NC	22.05	35.70	27.60	15.17	14.22	8.76	23.12	17.01	24.56
22	Piperonyl butoxide	51-03-06	liquid	NC	99.15	75.30	72.85	112.64	100.20	92.28	74.55	105.76	96.73
23	Polyethyleneglycol(PEG-40) hydrogenated castor oil	61788-85-0	viscous	NC	55.20	83.00	78.05	117.02	101.53	67.92	53.51	90.90	95.44
24	1-(4-Chlorophenyl)-3-(3,4-dichlorophenyl) urea	101-20-2	solid	NC	101.20	90.00	92.35	126.17	95.58	62.88	99.15	99.12	104.16
25	2,2'-[[3-Methyl-4-[(4-nitrophenyl)azo]-phenyl] imino]bis-ethanol	3179-89-3	solid	NC	33.45	23.25	36.60	58.94	56.09	66.75	35.76	52.64	72.35
26	2,2'-Methylene-bis-(6-(2H-benzotriazol-2-yl)-4-(1,1,3,3-tetramethylbutyl)-phenol)	103597-45-1	solid	NC	108.55	88.30	99.60	116.86	101.46	86.99	74.11	98.95	98.44
27	4,4'-Methylene bis-(2,6-di-tert-butylphenol)	118-82-1	solid	NC	107.40	97.60	99.80	127.42	99.09	62.34	76.91	103.06	101.09
28	Cellulose, 2-(2-hydroxy-3-(trimethylammonium)propoxy)ethyl ether chloride (91%)	68610-92-4	solid	NC	85.20	70.95	93.95	100.25	85.67	93.56	84.81	88.92	93.00
29	Potassium tetrafluoroborate	14075-53-7	solid	NC	91.30	83.90	96.35	95.67	97.85	101.90	84.49	103.36	101.99

30	Trisodium mono-(5-(1,2-dihydroxyethyl)-4-oxido-2-oxo-2,5-dihydro-furan-3-yl) phosphate	66170-10-3	solid	NC	9.60	5.90	20.60	19.58	28.19	14.15	2.02	7.65	6.60

Lab 1; Biosolution Co., Lab 2; Korea Conformity Laboratories, Lab 3; Biotoxtech Co..

Red cells; determined as irritants

The predictive capacity, based on 45% cutoff, of the respective laboratory analyses for a set of 30 reference chemicals obtained during reproducibility tests was comparable, with the sensitivity ranging from 89.6% to 93.3%, the accuracy from 75.9% to 80%, and the specificity from 62.2% to 66.7% (Table 12).

[Table 12] Predictive capacity based on "yes" or "no" classification

	PS	**Approach 1***				**Approach 2****	**Approach 3*****
		Lab 1	Lab 2	Lab 3	Total (270)		
Sensitivity	≥90%	93.3%	91.1%	84.4%	89.6%	91.1%	93.3%
Specificity	≥60%	60.0%	64.4%	62.2%	62.2%	62.2%	66.7%
Accuracy	≥75%	76.7 %	77.8%	73.3%	75.9%	76.7%	80.0%

*** Approach 1;** 270 runs were estimated independently, ****Approach 2;** based on the decision of 30 chemicals from the average cell viability from each laboratory (90 determinations**)**, *****Approach 3;** based on the decision of 30 chemicals from majority votes (30 determinations).

Lab 1; Biosolution Co., Lab 2; Korea Conformity Laboratories, Lab 3; Biotoxtech.

According to the different methods of approach (Approach 1, 270 runs were estimated independently; Approach 2, based on the decision of 30 chemicals from the average cell viability from each laboratory; Approach 3, based on the decision of 30 chemicals from majority votes), results indicated the sensitivity was barely met or just failed to meet the acceptance criteria given in the OECD GD216, which was due to the sporadic false predictions for sodium oxalate or 1,5-naphthalenediol.

6.2 Color Interference

Biosolution Co. has conducted the correction procedure for color interference with WST-1 as described in VII.1.3 for 30 reference chemicals to check the possibility of direct staining and WST-1 reactivity [Table 13]. Through the test, it was concluded that the test results were not needed to be corrected for color interferences since all four direct staining chemicals were determined to be irritants (which needed no correction). There were five non-irritants exhibiting reactivity, but they had only minimal impact on the decision.

[Table 13] Color interference check for 30 PS reference chemicals in Biosolution Co.

No.	**Chemical**	**CAS No.**	**Physical state**	**UN GHS**	**Uncorrected cell viability**			**Decision**	**Direct Staining**	**WST-1 reactivity**	**Correction**
					1 2017.04	**2** 2017.05	**3** 2017.06				
1	(Ethylenediamine-propyl)-trimethoxysilane	1760-24-3	liquid	Cat1	31.80	10.85	32.45	I		O	X
2	Methylthioglycolate	2365-48-	liquid	Cat1	21.30	32.10	22.35	I		O	X

3	Tetraethylene glycol diacrylate	2 17831-71-9	liquid	Cat1	2.30	3.10	2.65	I		O	X
4	1,2-Benzisothiazol-3(2H)-one	2634-33-5	solid	Cat1	4.00	0.65	0.95	I			
5	2,5-Dimethyl-2,5-hexanediol	110-03-2	solid	Cat1	0.45	0.55	0.55	I			
6	Disodium 2,2'-([1,1'-biphenyl]-4,4'-diyldivinylene)bis-(benzenesulphonate)	27344-41-8	solid	Cat1	16.00	10.20	30.75	I	O		X
7	Sodium oxalate	62-76-0	solid	Cat1	30.50	36.90	24.35	I			
8	2,4,11,13-Tetraazatetradecane-diimidamide,N,N''-bis(4-chlorophenyl)-3,12-diimino-,di-D-gluconate (20%,aqueous)	18472-51-0	liquid	Cat2A	1.90	1.85	1.75	I			
9	gamma-Butyrolactone	96-48-0	liquid	Cat2A	3.10	2.40	3.55	I		O	X
10	1,5-Naphthalenediol	83-56-7	solid	Cat2A	39.40	38.05	35.85	I	O	O	X
11	Sodium benzoate	532-32-1	solid	Cat2A	0.85	1.70	0.70	I			
12	2-Methyl-1-pentanol	105-30-6	liquid	Cat2B	2.55	1.65	9.15	I		O	X
13	Diethyl toluamide	134-62-3	liquid	Cat2B	0.85	1.30	4.85	I		O	X
14	1,4-Dibutoxy benzene	104-36-9	solid	Cat2B	97.60	73.25	96.65				
15	2,2-Dimethyl-3- methylenebicyclo [2.2.1] heptane	79-92-5	solid	Cat2B	31.80	9.90	27.05	I			
16	1-Ethyl-3- methylimidazolium ethylsulphate	342573-75-5	liquid	NC	55.15	54.45	60.65			O	- 4.31
17	2-Ethoxyethyl methacrylate	2370-63-0	liquid	NC	0.90	1.20	1.85	I		O	X
18	3-Phenoxybenzyl alcohol	13826-35-2	liquid	NC	9.80	5.50	25.00	I	O	O	X
19	4-(Methylthio)-benzaldehyde	3446-89-7	liquid	NC	10.50	27.30	22.55	I		O	X
20	Dipropyl disulphide	629-19-6	liquid	NC	46.45	51.60	55.20			O	-2.99
21	Ethyl thioglycolate	623-51-8	liquid	NC	22.05	35.70	27.60	I		O	X
22	Piperonyl butoxide	1951-03-06	liquid	NC	99.15	75.30	72.85			O	-0.11
23	Polyethyleneglycol(PEG-40) hydrogenated castor oil	61788-85-0	viscous	NC	55.20	83.00	78.05			O	0.20
24	1-(4-Chlorophenyl)-3- (3,4-dichlorophenyl) urea	101-20-2	solid	NC	101.20	90.00	92.35				
25	2,2'-[[3-Methyl-4-[(4-nitrophenyl)azo]-phenyl] imino]bis-ethanol	3179-89-3	solid	NC	33.45	23.25	36.60	I	O	O	X
26	2,2'-Methylene-bis-(6- (2H-benzotriazol-2-yl)-4-(1,1,3,3-tetramethylbutyl)- phenol)	103597-45-1	solid	NC	108.55	88.30	99.60			O	-2.28
27	4,4'-Methylene bis-(2,6-di-tert-butylphenol)	118-82-1	solid	NC	107.40	97.60	99.80				
28	Cellulose, 2-(2-hydroxy-3-(trimethylammonium)propoxy)ethyl ether chloride (91%)	68610-92-4	solid	NC	85.20	70.95	93.95				
29	Potassium tetrafluoroborate	14075-53-7	solid	NC	91.30	83.90	96.35				
30	Trisodium mono-(5-(1,2-dihydroxyethyl)-4-oxido-2-oxo-2,5-dihydro-furan-3-yl) phosphate	66170-10-3	solid	NC	9.60	5.90	20.60	I		O	X

*Red cell is irritant based on uncorrected cell viability. X, correction unnecessary

Other two laboratories also confirmed on their own that color intereference did not affect the decision

made with uncorrected viability as shown below (Table 14).

[Table 14] Color interference check for 30 PS reference chemicals in Korea Conformity Laboratories and Biotoxtech

No	Chemical	UN GHS	Korea Conformity Laboratories							Biotoxtech						
			Uncorrected cell viability			Pred.	Direct Stain	WST-1 React.	Corr.	Uncorrected cell viability			Pred.	Direct Stain	WST-1 React.	Corr.
			1 2017.07	2 2017.08	3 2017.09					1 2017.05	2 2017.07	3 2017.09				
1	(Ethylenediamine-propyl)-trimethoxysilane	Cat1	24.68	17.02	29.95	I				28.09	16.17	39.45	I			
2	Methylthioglycolate	Cat1	13.08	11.89	22.85	I		O	X	24.39	17.27	24.42	I		O	X
3	Tetraethylene glycol diacrylate	Cat1	2.41	1.78	2.17	I				3.57	3.50	3.15	I			
4	1,2-Benzisothiazol-3(2H)-one	Cat1	0.72	0.63	0.43	I				0.61	0.78	0.78	I			
5	2,5-Dimethyl-2,5-hexanediol	Cat1	0.51	0.67	0.29	I				5.83	0.49	1.15	I			
6	Disodium 2,2'-([1,1'-biphenyl]-4,4'-diyldivinylene)bis-(benzenesulphonate)	Cat1	6.44	8.65	2.69	I				3.23	12.52	25.58	I		O	X
7	Sodium oxalate	Cat1	38.09	18.81	32.17	I				21.87	46.96	33.31	I			
8	2,4,11,13-Tetraazatetradecane-diimidamide,N,N''-bis(4-chlorophenyl)-3,12-diimino-,di-D-gluconate (20%,aqueous)	Cat2A	1.95	1.24	11.78	I				2.33	2.05	1.58	I			
9	gamma-Butyrolactone	Cat2A	5.52	3.29	3.4	I	O		X	2.46	1.94	5.17	I			
10	1,5-Naphthalenediol	Cat2A	62.97	32.1	25.18	NI/I		O	0.15	50.87	56.59	80.43	NI	O	O	1.95
11	Sodium benzoate	Cat2A	0.85	1.5	0.52	I				0.32	0.85	4.04	I			
12	2-Methyl-1-pentanol	Cat2B	2.4	0.88	3.72	I				2.30	3.32	2.31	I			
13	Diethyl toluamide	Cat2B	2.75	5.2	1.11	I	O		X	4.33	2.19	8.66	I			
14	1,4-Dibutoxy benzene	Cat2B	103.18	102.98	105.63	NI				104.83	99.46	102.37	NI			
15	2,2-Dimethyl-3-methylenebicyclo [2.2.1] heptane	Cat2B	23.26	15.67	6.48	I				4.39	30.01	35.88	I			
16	1-Ethyl-3- methylimidazolium ethylsulphate	NC	68.21	59.46	60.01	NI				24.49	56.88	65.37	NI			
17	2-Ethoxyethyl methacrylate	NC	1.11	0.77	7.4	I	O		X	0.87	1.10	1.44	I			
18	3-Phenoxybenzyl alcohol	NC	16.1	26.4	7.41	I				0.73	20.89	15.57	I			
19	4-(Methylthio)-benzaldehyde	NC	9.95	5.76	68.59	I/NI				9.12	7.25	31.95	I			
20	Dipropyl disulphide	NC	69.7	8.62	14.74	I/NI				52.26	77.54	55.68	NI			
21	Ethyl thioglycolate	NC	15.17	14.22	8.76	I		O	X	23.12	17.01	24.56	I		O	X
22	Piperonyl butoxide	NC	112.64	100.2	92.28	NI				74.55	105.76	96.73	NI			
23	Polyethyleneglycol(PEG-40) hydrogenated castor oil	NC	117.02	101.53	67.92	NI				53.51	90.90	95.44	NI			
24	1-(4-Chlorophenyl)-3- (3,4-dichlorophenyl) urea	NC	126.17	95.58	62.88	NI				99.15	99.12	104.16	NI			
25	2,2'-[[3-Methyl-4-[(4-nitrophenyl)azo]-phenyl] imino]bis-ethanol	NC	58.94	56.09	66.75	NI	ND	ND		35.76	52.64	72.35	NI	ND	ND	
26	2,2'-Methylene-bis-(6- (2H-benzotriazol-2-yl)-4-(1,1,3,3-tetramethylbutyl)- phenol)	NC	116.86	101.46	86.99	NI				74.11	98.95	98.44	NI			
27	4,4'-Methylene bis-(2,6-di-tert-butylphenol)	NC	127.42	99.09	62.34	NI				76.91	103.06	101.09	NI			
28	Cellulose, 2-(2-hydroxy-3-(trimethylammonium)propoxy)ethyl ether chloride (91%)	NC	100.25	85.67	93.56	NI				84.81	88.92	93.00	NI			
29	Potassium tetrafluoroborate	NC	95.67	97.85	101.9	NI				84.49	103.36	101.99	NI			

30	Trisodium mono-(5-(1,2-dihydroxyethyl)-4-oxido-2-oxo-2,5-dihydro-furan-3-yl) phosphate	NC	19.58	28.19	14.15	I		O	X	2.02	7.65	6.60	I		O	X

ND, PS 25 substance was not tested due to limited availability. X, correction unnecessary

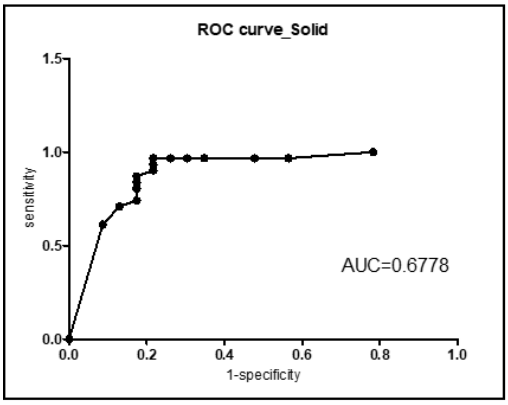
As shown in the results of Korea Conformity Laboratories and Biotoxtech, the direct staining chemicals and the chemicals with WST-1 reactivity were all irritants, which needed no correction. Chemcal No. 25, 2,2'-[[3-methyl-4-[(4-nitrophenyl)azo]-phenyl] imino]bis-ethanol was excluded in the test since the available amount was insufficient. Details on the color interference tests were provided as Appendix 12.

7. Optimization of Prediction Model with Separate Cut-offs for Liquids and Solids

The data for 141 reference chemicals in total (130 chemicals previously tested to evaluate predictive capacity of MCTT HCE^{TM} EIT, which were shown in section 3.1, nine reference chemicals in the original OECD GD 216 (OECD, 2015a) non-overlapped with 130 chemicals, and two new PS reference chemicals that were added in the revised OECD GD 216 in 2017 (OECD, 2017a) with known *in vivo* irritancy, composed of 87 liquids and 54 solids (80 irritants and 61 non-irritants) were evaluated to establish the prediction model of MCTT HCE^{TM} EIT with ROC analysis.

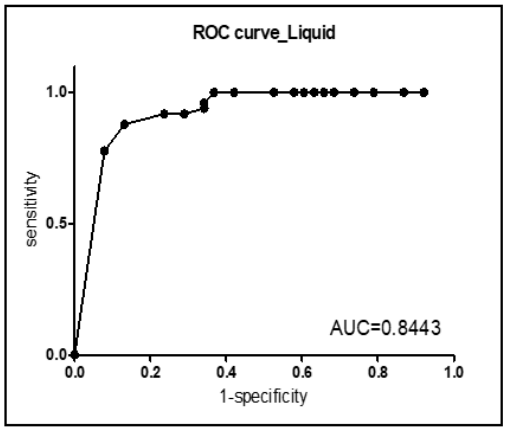
The optimal cutoff value was 40-45% when considering both liquid and solid chemical together but different cutoff values for liquid and solid may improve the predictive capacity. Based on that, applying different cutoff values for liquid and solid could be another strategy to increase the predictive capacity of the MCTT HCE^{TM} EIT. To confirm the validity of the idea, ROC curves were redrawn separately for liquid and solid as shown in Figure 6, and WLR, BLR and predictive capacity for the 30 reference chemicals were re-assessed as well.

Solid only



	Cut-off	sen.	spc.	acc.	1-spc.
	5	0.613	0.913	0.741	0.087
	10	0.710	0.870	0.778	0.130
	15	0.742	0.826	0.778	0.174
	20	0.806	0.826	0.815	0.174
	25	0.839	0.826	0.833	0.174
	30	0.839	0.826	0.833	0.174
	35	0.871	0.826	0.852	0.174
	40	0.871	0.826	0.852	0.174
	45	0.871	0.826	0.852	0.174
	50	0.903	0.783	0.852	0.217
	55	0.935	0.783	0.870	0.217
SOLID	60	0.968	0.783	0.889	0.217
	65	0.968	0.739	0.870	0.261
	70	0.968	0.739	0.870	0.261
	75	0.968	0.696	0.852	0.304
	80	0.968	0.696	0.852	0.304
	85	0.968	0.652	0.833	0.348
	90	0.968	0.522	0.778	0.478
	95	0.968	0.435	0.741	0.565
	100	1.000	0.217	0.667	0.783

Liquid only



	Cut-off	sen.	spc.	acc.	1-spc.
	5	0.776	0.921	0.839	0.079
	10	0.878	0.868	0.874	0.132
	15	0.918	0.763	0.851	0.237
	20	0.918	0.711	0.828	0.289
	25	0.939	0.658	0.816	0.342
	30	0.959	0.658	0.828	0.342
LIQUID	35	1.000	0.632	0.839	0.368
	40	1.000	0.579	0.816	0.421
	45	1.000	0.579	0.816	0.421
	50	1.000	0.474	0.770	0.526
	55	1.000	0.421	0.747	0.579
	60	1.000	0.395	0.736	0.605
	65	1.000	0.395	0.736	0.605
	70	1.000	0.368	0.724	0.632
	75	1.000	0.342	0.713	0.658
	80	1.000	0.316	0.701	0.684
	85	1.000	0.263	0.678	0.737
	90	1.000	0.211	0.655	0.789
	95	1.000	0.132	0.621	0.868
	100	1.000	0.079	0.598	0.921

[Figure 6] ROC curves of the predictive capacity for 54 solids and 87 liquids

According to ROC analysis, 35% cutoff value is the optimal for liquids while 60% cutoff is the optimal for solids. By applying the optimal cutoff values separately for liquids and solids, the predictive capacity for 141 substances was improved as shown below (Table 15).

[Table 15] Re-assessment of predictive capacity for 141 chemicals based on a new prediction model with the optimized cut-off values for liquids and solids

		Total (141)		Liquid (87)		Solid (54)		PS
		I	NI	I	NI	I	NI	criteria
New Model (liquid 35% / solid 60%)	I	79	1	49	0	30	1	
	NI	19	42	14	24	5	18	
	total		141		87		54	
	Sensitivity	**98.8% (▲)**		**100%**		**96.8%(▲)**		90%
	Specificity	**68.9% (▲)**		**63.2% (▲)**		**78.3% (▼)**		60%
	Accuracy	**85.8% (▲)**		**83.9% (▲)**		**88.9%(▲)**		75%
Old Model (single cut-off, 45%)	I	76	4	49	0	27	4	
	NI	20	41	16	22	4	19	
	total		141		87		54	
	Sensitivity	95.0%		100%		87.1%		90%
	Specificity	67.2%		57.9%		82.6%		60%
	Accuracy	83.0%		81.6%		85.2%		75%

(▲) increase or (▼) decrease on predictive capacity based on new model vs old model

7.1 WLR and BLR as Separate Cut-offs for Liquids (35%) and Solids (60%)

In addition to redrawing ROC curves for liquid and solid separately, re-assessment of WLR/BLR on the 30 reference chemicals was done as well according to the new model, which is to apply separate cut-offs for liquids (35%) and solids (60%). The results are shown in Table 16.

[Table 16] Re-assessment of WLR/BLR for old and new 30 reference chemicals with new prediction model based on the separate cut-offs for liquids and solids

Prediction Model	**New 30 PS**				**Old 30 PS**				**PS Criteria (WLR/BLR)**
	WLR			**BLR**	**WLR**			**BLR**	
	BS	**KCL**	**BTT**		**BS**	**KCL**	**BTT**		
New Model	96.7%	90.0%	90.0%	93.3%	96.7%	86.7%	86.7%	90.0%	90%/85%
Old Model	100.0%	90.0%	90.0%	90.0%	100.0%	90.0%	90.0%	90.0%	

*Red, failed to meet the PS criteria

7.2 Predictive Capacity as Separate Cut-offs for Liquids (35%) and Solids (60%)

In the same vein, the new prediction model was applied to estimate the predictive capacity for 30 substances (new and old PS 30 reference chemical sets) and the results of the re-assessment are shown below (Table 17). Predictive capacity for the new PS 30 substances set is comparable between the two prediction models while the new prediction model with separate cut-offs for liquids and solids is better in terms of sensitivity for the old 30 PS set.

[Table 17] Re-assessment of predictive capacity for old and new PS 30 chemicals sets for Approach 1 based on a new prediction model with the separate cutoff values for liquids and solids

PS Criteria			New 30 PS				Old 30 PS			
			BS	KCL	BTT	Total (270)	BS	KCL	BTT	Total (270)
New Model	90%	Sensitivity	93.3%	93.3%	91.1%	92.6%	93.3%	91.1%	88.9%	91.1%
	60%	Specificity	62.2%	60.0%	60.0%	60.7%	62.2%	60.0%	60.0%	60.7%
	75%	Accuracy	77.8%	76.7%	75.6%	76.7%	77.8%	75.6%	74.4%	75.9%
Old Model	90%	Sensitivity	93.3%	93.3%	91.1%	92.6%	93.3%	91.1%	84.4%	89.6%
	60%	Specificity	60.0%	64.4%	62.2%	62.2%	60.0%	64.4%	62.2%	62.2%
	75%	Accuracy	76.7%	78.9%	76.7%	77.4%	76.7%	77.8%	73.3%	75.9%

*Red, failed to meet the PS criteria

IX. Discussion

1. In Consideration of Invalid Runs

In accordance with the re-test criteria #4, most of the re-tests were due to the occurrence of the borderline results, namely the viability fell between 40% and 50%. Of the total 270 determinations, re-tests were done 19 times (8 times in Biosolution Co. (all criteria #4), none in Korea Conformity Laboratories, and 11 times in Biotoxtech Co. (3 times criteria #3[difference over 20% between duplicate wells], and others criteria #4)). Though, all these invalid results were resolved after the first or second rounds of re-tests in each laboratory. As a result, complete runs of three repetitions were accomplished for 30 PS chemicals by all three laboratories.

2. Reliability

WLRs were 100% at Lab 1, 90.0% at Lab 2, and 90.0% at Lab 3, thus meeting the requirements specified in the TG 492 performance standards, which is to be ≥ 90%. BLR for all three participating laboratories was 90.0%, thus meeting the ≥ 85% criteria as specified in the TG 492 performance standards. The results demonstrated that the robustness and reliability of the test method is sufficient to meet the performance standards in OECD GD 216.

3. Predictive Capacity

All three laboratories completed three valid runs successfully. Sensitivity was determined at each laboratory in the range from 84.4% to 93.3%. The results showed the sensitivity of 89.6% when all 270 individual runs were taken into account assessing the predictive capacity, and 91.1% sensitivity when considered 90 determinations (decisions made for 30 chemicals based on the average cell viability in each laboratory). When 30 determinations (decisions made for 30 chemicals based on majority votes among laboratories) were considered, the sensitivity was 93.3% which met the sensitivity criterion stated in the OECD GD 216 performance standards, which is ≥ 90%. (Table 12). Specificity was determined from 60.0% to 64.4% at each laboratory when individual runs were considered, resulting in 62.2% specificity in a total of 270 runs. When the predictive capacity was assessed based on the decision of 30 chemicals from the average cell viability at each laboratory, specificity was 62.2% (90 determinations). Specificity estimated based on the decision of 30 chemicals from majority votes was 66.7%, meeting a specificity criterion of ≥ 60% as stated in the OECD GD 216 performance standards (OECD, 2015a). Likewise, the accuracy was determined from 73.3% to 77.8% at each laboratory when individual runs were considered, resulting in 75.9% accuracy in a total of 270 runs. When the predictive capacity was assessed based on the decision of 30 chemicals from the average cell viability at each laboratory, accuracy was 76.7% (90 determinations in total). Accuracy estimated based on the decision of 30 chemicals from majority votes was 80.0% meeting an accuracy criterion of ≥ 75% as stated in the OECD GD 216 performance standards.

Almost all UN GHS Category 1 or 2 chemicals were correctly determined as irritants with the exception of the intrinsically false negative determination of 1,4-dibutoxy benzene (No. 14, Category 2B) and sporadic occurrence of false positive determination of sodium oxalate (No. 7, Category 1, in one run by Lab 3) and 1,5-naphthalenediol (No. 10, Category 2A, in one run by Lab 2 and in all three runs by Lab 3). 1,4-Dibutoxy benzene was reported as a false negative in the EpiOcular™ EIT (OECD, 2015c) and 1,5-naphthalenediol appears as a borderline irritant which is in line with the case in SkinEthic HCE EIT (Alepee *et al.*, 2016).

Overview of wrongly classified chemicals is shown in Table 18. 8 chemicals indicated false positives and 3 chemicals indicated false negatives. Among the cases of false positives, 2-ethoxyethyl methacrylate (No. 17), 3-phenoxybenzyl alcohol (No. 18), ethyl thioglycolate (No. 21), and trisodium mono-(5-(1,2-dihydroxyethyl)-4-oxido-2-oxo-2,5-dihydro-furan-3-yl) phosphate (No. 30) indicated false positives in all runs by all three laboratories. Other false positive cases were 1-ethyl-3-methylimidazolium ethylsulphate (No. 16, in one run by Lab 3), 4-(methylthio)-benzaldehyde (No. 19, in all runs by Lab 1 and Lab 3, and in two runs by Lab 2), dipropyl disulphide (No. 20, in two runs in Lab 2), and 2,2'-[[3-methyl-4-[(4-nitrophenyl)azo]-phenyl]imino]bis-ethanol (No. 25, in three runs by Lab 1 and one run in Lab 3). False negative cases were 1,4-dibutoxy benzene (No. 14, indicated false negative in all runs by all three laboratories), sodium oxalate (No. 7, in one run by Lab 3), and 1,5-naphthalenediol (No. 10, in one run by Lab 2 and three runs by Lab 3). 4-(Methylthio)-benzaldehyde (No. 19) was determined to be an irritant in nearly all valid runs, and this appeared to be an inherent issue in the MCTT HCE™ EIT. Chemicals No. 14, 17, 18, 21, 25 and 30 were falsely predicted in the EpiOcular™ EIT as well. False predictions for No. 16 and 20 appeared to be isolated cases.

[Table 18] Overview of wrong classified chemicals in the PS

PS No.	Chemical	Physical state	UN GHS	Lab 1			Lab 2			Lab 3		
				1	2	3	1	2	3	1	2	3
7	Sodium oxalate	solid	Cat1	TP	TP	TP	TP	TP	TP	TP	FN	TP
10	1,5-Naphthalenediol	solid	Cat2A	TP	TP	TP	FN	TP	TP	FN	FN	FN
14	1,4-Dibutoxy benzene	solid	Cat2B	FN	FN	FN	FN	FN	FN	FN	FN	FN
16	1-Ethyl-3-methylimidazolium ethylsulphate	liquid	NC	TN	TN	TN	TN	TN	TN	FP	TN	TN
17	2-Ethoxyethyl methacrylate	liquid	NC	FP	FP	FP	FP	FP	FP	FP	FP	FP
18	3-Phenoxybenzyl alcohol	liquid	NC	FP	FP	FP	FP	FP	FP	FP	FP	FP
19	4-(Methylthio)-benzaldehyde	liquid	NC	FP	FP	FP	FP	FP	TN	FP	FP	FP
20	Dipropyl disulphide	liquid	NC	TN	TN	TN	TN	FP	FP	TN	TN	TN
21	Ethyl thioglycolate	liquid	NC	FP	FP	FP	FP	FP	FP	FP	FP	FP
25	2,2'-[[3-Methyl-4-[(4-nitrophenyl)azo]-phenyl]imino]bis-ethanol	solid	NC	FP	FP	FP	TN	TN	TN	FP	TN	TN
30	Trisodium mono-(5-(1,2-dihydroxyethyl)-4-oxido-2-oxo-2,5-dihydro-furan-3-yl) phosphate	solid	NC	FP	FP	FP	FP	FP	FP	FP	FP	FP

TP; True positive, TN; True negative, FP; False positive, FN; False negative.
Lab 1; Biosolution Co., Lab 2; Korea Conformity Laboratories, Lab 3; Biotoxtech Co..

3.1 Predictive capacity for 130 chemicals (selected from ECETOC database)

To further assess the predictive capacity of the MCTT HCE^{TM} EIT, the data for 130 reference chemicals with known *in vivo* irritancy, composed of 81 liquids and 49 solids (76 irritants and 54 non-irritants), which were selected from the ECETOC database, were analyzed. The composition of the 130 chemicals was as below (Table 19).

[Table 19] Composition of the 130 chemicals used in the evaluation of the predictive capacity of the MCTT HCE^{TM} EIT

	Total	Liquid (81)	Solid (49)
Cat 1	38	21	17
Cat 2A/2B	38	26	12
NC	54	34	20

NC, No Category.

The tests were conducted independently from the management and guidance of the current VMT. The data were re-analyzed in compliance with protocol 1.6. 81 liquids were tested according to protocol 1.2(1.4), which is compatible to protocol 1.6, by AmorePacific, Biotoxtech Co., or Korea Testing & Research Institute (KTR) without coding. 49 solids were tested based on protocol 1.6 by Ewha Womans

University, Biosolution Co., or KTR in a coded fashion. The test results were uncorrected for color interference. As a result, sensitivity was 96.1% and specificity was 72.2%. The overall accuracy reached 86.2% based on 45% cutoff (Table 20).

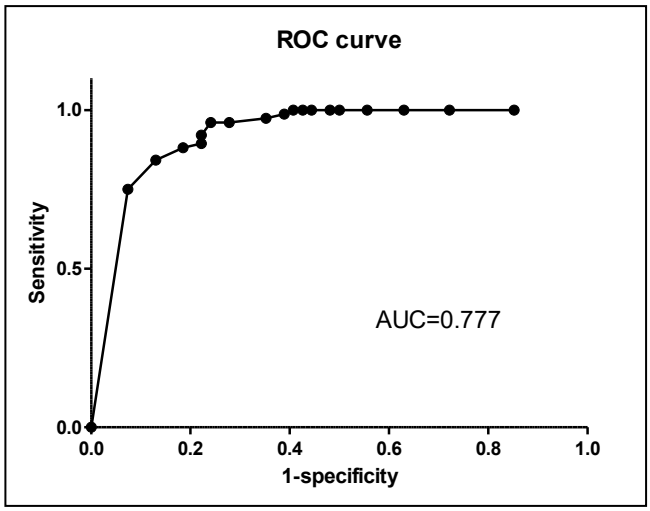
Three irritants were classified as non-irritants as false negative, while 15 non-irritants were determined as irritants (false positives). Overall, MCTT HCE^{TM} EIT showed higher sensitivity for liquids than solids while specificity was better for solids. Accuracy was 85.2-87.8%, which is comparable between liquids and solids.

[Table 20] Predictive capacity for 130 chemicals

	PS criteria	Total (130)		Liquid (81)		Solid (49)	
		I	NI	I	NI	I	NI
I		73	3	47	0	26	3
NI		15	39	12	22	3	17
total		130		81		49	
Sensitivity	≥ 90%	96.1%		100.0%		89.7%	
Specificity	≥ 60%	72.2%		64.7%		85.0%	
Accuracy	≥ 75%	86.2%		85.2%		87.8%	

PS; performance standards (OECD, 2015a).

The optimal cutoff to maximize the predictive capacity of the MCTT HCE^{TM} EIT was estimated with the viability data for 130 reference chemicals through ROC (receiver operative characteristics) curve analysis as below in Figure 7. Based on the statistical analysis, 45% cutoff value was determined to be the optimal for the maximization of sensitivity as well as accuracy for 130 substances.



[Figure 7] ROC curve of the predictive capacity for 130 chemicals

3.2 Predictive capacity for 2 PS substances newly included in revised OECD GD216

In 2017, OECD GD 216 has been revised, and two PS substances, tetraethylene glycol diacrylate (No. 3, Cat 1, liquid) and 1,5-naphthalenediol (No. 10, Cat 2A, solid) were replaced by 2-amino-3-hydroxy pyridine (No. 3, Cat 2A, solid) and 2-hydroxyethyl acrylate (No. 10, Cat 1, liquid). These two new PS substances were tested by three participating laboratories under coded states in three repeats completing a new set of 30 PS substances in the revised OECD GD 216 (OECD, 2017a). The results are in Table 21,

[Table 21] Individual test results for 30 reference chemicals in revised OECD GD 216 conducted three times by three participating laboratories

No	Chemical	CAS No.	Physical state	UN GHS	Lab 1			Lab 2			Lab 3		
					1	2	3	1	2	3	1	2	3
1	(Ethylenediamine-propyl)-trimethoxysilane	1760-24-3	liquid	Cat1	31.80	10.85	32.45	24.68	17.02	29.95	28.09	16.17	39.45
2	Methylthioglycolate	2365-48-2	liquid	Cat1	21.30	32.10	22.35	13.08	11.89	22.85	24.39	17.27	24.42
3	2-Amino-3-hydroxy pyridine	16867-03-1	solid	Cat2A	26.90	15.95	18.95	17.05	12.25	2.45	27.70	27.65	13.40
4	1,2-Benzisothiazol-3(2H)-one	2634-33-5	solid	Cat1	4.00	0.65	0.95	0.72	0.63	0.43	0.61	0.78	0.78
5	2,5-Dimethyl-2,5-hexanediol	110-03-2	solid	Cat1	0.45	0.55	0.55	0.51	0.67	0.29	5.83	0.49	1.15
6	Disodium 2,2'-([1,1'-biphenyl]-4,4'-diyldivinylene)bis-(benzenesulphonate)	27344-41-8	solid	Cat1	16.00	10.20	30.75	6.44	8.65	2.69	3.23	12.52	25.58
7	Sodium oxalate	62-76-0	solid	Cat1	30.50	36.90	24.35	38.09	18.81	32.17	21.87	46.96	33.31
8	2,4,11,13-Tetraazatetradecane-diimidamide,N,N''-bis(4-chlorophenyl)-3,12-diimino-,di-D-gluconate (20%,aqueous)	18472-51-0	liquid	Cat2A	1.90	1.85	1.75	1.95	1.24	11.78	2.33	2.05	1.58
9	gamma-Butyrolactone	96-48-0	liquid	Cat2A	3.10	2.40	3.55	5.52	3.29	3.40	2.46	1.94	5.17
10	2-Hydroxyethyl acrylate	818-61-1	liquid	Cat1	15.9	1.05	12.3	0.5	0.55	1.65	2.85	8.05	4.85
11	Sodium benzoate	532-32-1	solid	Cat2A	0.85	1.70	0.70	0.85	1.50	0.52	0.32	0.85	4.04
12	2-Methyl-1-pentanol	105-30-6	liquid	Cat2B	2.55	1.65	9.15	2.40	0.88	3.72	2.30	3.32	2.31
13	Diethyl toluamide	134-62-3	liquid	Cat2B	0.85	1.30	4.85	2.75	5.20	1.11	4.33	2.19	8.66
14	1,4-Dibutoxy benzene	104-36-9	solid	Cat2B	97.60	73.25	96.65	103.18	102.98	105.63	104.83	99.46	102.37
15	2,2-Dimethyl-3-methylenebicyclo [2.2.1] heptane	79-92-5	solid	Cat2B	31.80	9.90	27.05	23.26	15.67	6.48	4.39	30.01	35.88
16	1-Ethyl-3-methylimidazolium ethylsulphate	342573-75-5	liquid	NC	55.15	54.45	60.65	68.21	59.46	60.01	24.49	56.88	65.37
17	2-Ethoxyethyl methacrylate	2370-63-0	liquid	NC	0.90	1.20	1.85	1.11	0.77	7.40	0.87	1.10	1.44
18	3-Phenoxybenzyl alcohol	13826-35-2	liquid	NC	9.80	5.50	25.00	16.10	26.40	7.41	0.73	20.89	15.57
19	4-(Methylthio)-benzaldehyde	3446-89-7	liquid	NC	10.50	27.30	22.55	9.95	5.76	68.59	9.12	7.25	31.95
20	Dipropyl disulphide	629-19-6	liquid	NC	46.45	51.60	55.20	69.70	8.62	14.74	52.26	77.54	55.68
21	Ethyl thioglycolate	623-51-8	liquid	NC	22.05	35.70	27.60	15.17	14.22	8.76	23.12	17.01	24.56
22	Piperonyl butoxide	51-03-06	liquid	NC	99.15	75.30	72.85	112.64	100.20	92.28	74.55	105.76	96.73

23	Polyethyleneglycol(PEG-40) hydrogenated castor oil	61788-85-0	viscous	NC	55.20	83.00	78.05	117.02	101.53	67.92	53.51	90.90	95.44
24	1-(4-Chlorophenyl)-3-(3,4-dichlorophenyl) urea	101-20-2	solid	NC	101.20	90.00	92.35	126.17	95.58	62.88	99.15	99.12	104.16
25	2,2'-[[3-Methyl-4-[(4-nitrophenyl)azo]-phenyl] imino]bis-ethanol	3179-89-3	solid	NC	33.45	23.25	36.60	58.94	56.09	66.75	35.76	52.64	72.35
26	2,2'-Methylene-bis-(6-(2H-benzotriazol-2-yl)-4-(1,1,3,3-tetramethylbutyl)-phenol)	103597-45-1	solid	NC	108.55	88.30	99.60	116.86	101.46	86.99	74.11	98.95	98.44
27	4,4'-Methylene bis-(2,6-di-tert-butylphenol)	118-82-1	solid	NC	107.40	97.60	99.80	127.42	99.09	62.34	76.91	103.06	101.09
28	Cellulose, 2-(2-hydroxy-3-(trimethylammonium)propoxy)ethyl ether chloride (91%)	68610-92-4	solid	NC	85.20	70.95	93.95	100.25	85.67	93.56	84.81	88.92	93.00
29	Potassium tetrafluoroborate	14075-53-7	solid	NC	91.30	83.90	96.35	95.67	97.85	101.90	84.49	103.36	101.99
30	Trisodium mono-(5-(1,2-dihydroxyethyl)-4-oxido-2-oxo-2,5-dihydro-furan-3-yl) phosphate	66170-10-3	solid	NC	9.60	5.90	20.60	19.58	28.19	14.15	2.02	7.65	6.60

Lab 1; Biosolution Co., Lab 2; Korea Conformity Laboratories, Lab 3; Biotoxtech Co..

Red cells; determined as irritants.

Replacement of two PS substances improved the predictive capacity significantly as shown in Table 22.

[Table 22] Assessment of predictive capacity with the new PS 30 chemicals set

	PS criteria	**Approach 1***				**Approach 2****	**Approach 3*****
		BS	Korea Conformity Laboratories	BTT	Total (270)		
Sensitivity	90%	**93.3%**	**93.3%** (91.1%)	**91.1%** (84.4%)	**92.6%** (89.6%)	**93.3%** (91.1%)	**93.3%**
Specificity	60%	**60.0%**	**64.4%**	**62.2%**	**62.2%**	**62.2%**	**66.7%**
Accuracy	75%	**76.7 %**	**78.9%** (77.8%)	**76.7%** (73.3%)	**77.4%** (75.9%)	**77.8%** (76.7%)	**80.0%**

***Approach 1;** 270 runs were estimated independently, ****Approach 2;** based on the decision of 30 chemicals from the average cell viability from each laboratory (90 determinations), *****Approach 3;** based on the decision of 30 chemicals from majority votes (30 determinations). Lab 1; Biosolution Co., Lab 2; Korea Conformity Laboratories, Lab 3; Biotoxtech. Numbers inside the parenthesis are values of the old PS 30 substance, which have been improved with new PS 30 set.

3.3 Predictive capacity of MCTT HCE™ EIT for 141 chemicals in total

The data for 141 reference chemicals in total (130 chemicals previously tested to evaluate predictive capacity of MCTT HCE™ EIT, which were shown in section 3.1, nine reference chemicals in the original

OECD GD 216 (OECD, 2015a) non-overlapped with 130 chemicals, and two new PS reference chemicals that were added in the revised OECD GD 216 in 2017 with known *in vivo* irritancy, composed of 87 liquids and 54 solids (80 irritants and 61 non-irritants) were evaluated to assess the predictive capacity of MCTT HCE™ EIT. The results shown below indicated that MCTT HCE™ EIT has performance that meets the PS criteria (Table 23).

[Table 23] Assessment of predictive capacity with 141 chemicals in total

	Total (141)		**Liquid (87)**		**Solid (54)**		**PS**
	I	**NI**	**I**	**NI**	**I**	**NI**	**criteria**
I	79	1	49	0	30	1	
NI	19	42	14	24	5	18	
total	141		87		54		
Sensitivity	**95.0%**		100.0%		87.1%		90%
Specificity	**67.2%**		57.9%		82.6%		60%
Accuracy	**83.0%**		81.6%		85.2%		75%

3.4 Predictive capacity of MCTT HCE™ EIT for 113 credible reference substances after removing those "should not be selected for validation study" chemicals from 141 chemicals

While the reference chemicals were selected from credible sources, a recent study by Barroso et al. (2017) suggested that some chemicals should not be used for validation study based on the criteria they established. Comparison of 141 chemicals with the list provided in Barroso et al. (2017) indicated that 28 chemicals should not be used in the validation test. After removing those chemicals, 113 substances remained, with which the predictive capacity was re-assessed. Despite of excluding those chemicals that "should not be used", MCTT HCE™ EIT showed predictive capacity meeting the criteria of PS (Table 24).

[Table 24] Assessment of predictive capacity with 113 credible reference substances

	Total (113)		Liquid (67)		Solid (46)		PS criteria
	I	NI	I	NI	I	NI	
I	50	4	30	0	20	4	
NI	20	39	16	21	4	18	
total	113		67		46		
Sensitivity	92.6%		100%		83.3%		90%
Specificity	66.1%		56.8%		81.8%		60%
Accuracy	78.8%		76.1%		82.6%		75%

3.5 Weighted approach in assessing predictive capacity of MCTT HCE™ EIT

OECD GD216 (stated in paragraph 25 as below) advises to use weighted approach in the assessment

of predictive capacity.

> *"28. The calculation of predictive capacity (i.e., sensitivity, false negatives, specificity, false positives and accuracy) should be done using all qualified tests obtained for each Reference Chemical in each of at least three laboratories. The calculations should be based on the individual predictions of each qualified test for each Reference Chemical in each laboratory and neither on the arithmetic mean values of viability over the different qualified tests performed nor on the mode of all predictions obtained (or any other procedure used to summarize the multiple test results obtained into a single prediction per Reference Chemical). The predictive capacity should be determined using a weighted calculation in which the final outcome of each individual qualified test obtained for each Reference Chemical (from all laboratories participating in the validation study) is captured as an independent prediction in the calculations and correction factors are applied so that all Reference Chemicals have an equal weight in the calculations, even if it wasn't possible to obtain the same number of qualified tests for all Reference Chemicals during the validation study after re-testing (see paragraph 29). In summary, the "classified" and "not-classified" predictions for each Reference Chemical (obtained by the various laboratories participating in the study) should be divided by the total number of available predictions to determine the number of correct and under- or over-predictions for that chemical (as fractions of 1) and these should be used to calculate sensitivity, false negatives, specificity, false positives and accuracy so that all chemicals contribute with an equal weight of 1 in the calculations."*

As such, the predictive capacity of MCTT HCE^{TM} EIT can be re-assessed with the weighted approach for 141 chemicals since some chemicals have been tested more than once, but the number of tests was not same for all chemicals. As shown in Table 25, the predictive capacity of MCTT HCE^{TM} EIT met the criteria of PS regardless of weighting although new prediction model gave better results.

[Table 25] Re-assessment of predictive capacity using weighted approach for 141 chemicals

Prediction model/ Predictive capacity		PS criteria	**Weighted**			**Unweighted**		
			Total (141)	Liquid (87)	Solid (54)	Total (141)	Liquid (87)	Solid (54)
New Model	Sensitivity	90%	97.6%	98.6%	96.1%	98.8%	100%	96.8%
	Specificity	60%	68.5%	62.0%	79.2%	68.9%	63.2%	78.3%
	Accuracy	75%	85.0%	82.6%	88.9%	85.8%	83.9%	88.9%
Old Model	Sensitivity	90%	95.3%	99.5%	88.5%	95.0%	100%	87.1%
	Specificity	60%	65.0%	55.6%	80.7%	67.2%	57.9%	82.6%
	Accuracy	75%	82.2%	80.3%	85.2%	83.0%	81.6%	85.2%

*Red, failed to meet the PS criteria

3.6 Color Interference

Color interference is a potential issue for all formazan-based colorimetric viability assays. Even though Biosolution Co. (Lab 1) ensured that no correction was necessary for 30 PS substances, there remains a concern for other pool of chemicals. Korea Conformity Laboratories and Biotoxtech also

conducted color interference tests through which it was confirmed that no correction was necessary for 30 PS substances. There are some discrepancy in detecting color interference across laboratories which are attributable to adopting a different vessel for WST-1 and chemical mixing. To reduce variation in color interference tests, the protocol was revised that Eppendorf tube shall be used. In order to produce sufficient data for the color interference, Biosolution Co. checked color interference for 130 chemicals and newly included 2 PS substances. Among non-irritants from 130 chemicals, 13 chemicals were shown to have potentials for direct staining and determined as non-irritants by uncorrected viability. For WST-1 reactivity, 13 chemicals showed potential issues and were determined to be non-irritants by uncorrected viability. However, after applying correction procedure described in section VII. 1.3, it turned out that no correction was necessary. One of the newly included PS substances, tetraethylene glycol diacrylate (No. 3, Cat 1, liquid) showed result of direct staining, but it was determined as irritant, indicating that no correction was necessary.

In addition to viability-based correction procedure, HPLC analysis of formazan product after separating from colorful chemicals on column may be applied to do color correction. Preliminary test suggested that HPLC analysis can be used properly as color correction method, instead of OD measurement-based correction procedure. Details of color interference study are included in Appendix 12.

4. Similarity with the OECD TG 492 VRM

The VMT considered the MCTT HCE^{TM} EIT to be functionally and histologically similar to RhCE EIT VRMs in OECD TG 492. With the 30 reference chemicals from the Performance Standards, the MCTT HCE^{TM} EIT gave some false negatives and false positives to the extent comparable to the VRMs. These results suggest that the MCTT HCE^{TM} EIT has a predictive capacity comparable to that of the VRMs.

5. Limitation of the MCTT HCE^{TM} EIT

The VMT considers that, as similar with other EIT utilizing RhCE, a limitation of the MCTT HCE^{TM} EIT is that it does not allow discrimination between eye irritation/reversible effects on the eye (Category 2) and serious eye damage/irreversible effects on the eye (Category 1), nor between eye irritants (optional Category 2A) and mild eye irritants (optional Category 2B), as defined by UN GHS. For these purposes, further testing with other in vitro test methods is required.

X. Conclusion

This validation study aimed to determine if the MCTT HCE^{TM} EIT is capable of fulfilling the performance standards stipulated in OECD TG 492 for similar or modified *in vitro* RhCE EIT methods based on the EpiOcular^{TM} EIT. The assessment of reliability and relevance of the test method was performed using the 30 test chemicals selected from the reference chemicals listed in the performance standards, OECD GD 216 and additional chemicals to further assess predictive capacity. Having achieved

a WLR of from 90% to 100% in all three participating laboratories and a BLR of 90% in the three participating laboratories, the MCTT HCE™ EIT was considered to satisfy the criteria of performance standards, OECD GD 216.

The MCTT HCE™ EIT demonstrated a predictive capacity with an overall accuracy from 75.9% to 80%, an overall sensitivity from 89.6% to 93.3% and an overall specificity from 62.2% to 66.7% for the old set of 30 reference chemicals, depending on the estimation approaches. With a new set of 30 PS substances published in 2017, the MCTT HCE™ EIT demonstrated a predictive capacity with an overall accuracy from 77.4% to 80%, an overall sensitivity from 92.6% to 93.3% and an overall specificity from 62.2% to 66.7%. In additional predictive capacity analysis, a sensitivity of 95.0%, a specificity of 67.2% and an accuracy of 83.0% were obtained for the 141 chemicals, thereby meeting the criteria of 75% for accuracy, 90% for sensitivity, and 60% for specificity stated in the OECD GD 216 performance standards. Application of the new prediction model, i.e., separate cutoffs for liquid (35%) and solid (60%) improved predictive capacity considerably. The revision of protocol from 1.6 to 1.7 has been done to reflect the new prediction model. Collectively, these results suggest that the MCTT HCE™ EIT may be included as an additional VRM in OECD TG 492 for predicting the eye irritation potential of chemicals.

XI. Acknowledgements

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Annex 2: The peer-review report of the validation, coordinated by the Korean Centre for the Validation of Alternative Methods (KoCVAM).

Peer-Review of the Performance-based Validation Study on the MCTT HCE™ Eye Irritation Test (EIT) as a me-too test method according to OECD GD 216 and falling within the OECD TG 492

Date: 16 August 2018

Summary

The MCTT HCE™ Eye Irritation Test (EIT) method developed by Bio Solution (Korea) is proposed as a similar assay to the validated reference reconstructed human cornea-like epithelium (RhCE) test methods falling within the OECD TG 492. The method has undergone a performance standard (PS)-based validation study for eye hazard testing according to OECD GD 216. The assay underwent an independent peer-review coordinated by the Korean Centre for the Validation of Alternative Methods (KoCVAM), Keimyung University and SeCAM. The similarity of the me-too assay to the validated reference method, as well as its scientific validity was assessed by an international Peer Review Panel (PRP) composed of:

- Els Adriaens (Adriaens consulting, Belgium)
- Chantra Eskes (SeCAM, Switzerland)
- Kristina Kejlova (National Institute of Public Health, Czech Republic)
- Bae-Hwan Kim (Keimyung University, South Korea)
- Hajime Kojima (JaCVAM, NIHS, Japan)
- Jill Merrill (US FDA, USA)
- Uwe Pfannenbecker (Beiersdorf, Germany)

The criteria for peer-review evaluation were prepared by SeCAM and were revised by the PRP members. The non-governmental PRP members provided a declaration of interest that can be made available upon request. The peer-review took place from March to July 2018, and a total of four teleconferences took place as follows:

- 13 April 2018: presentation of the method by the test method developer and preliminary questions from the PRP;
- 11 June 2018: PRP draft evaluation of the method in the absence of the test method developer;
- 12 July 2018: answers to additional questions raised by the PRP by the test method developer;
- 26 July 2018: finalization of the PRP evaluation of the test method.

Based on its evaluation (see detailed criteria and evaluation below), the PRP is of the opinion that the information made available to them do support the scientific similarity of the MCTT HCE™ EIT to the validated reference methods both in terms of the essential test method components and of assay performance regarding its reproducibility and predictive capacity as described within the GD 216.

Evaluation criterion 1:

Rationale for the test method, including a description of the advantages of the similar or modified test method in terms of i) mechanistic advantages, applicability, predictive capacity, technical advances, reduction in hazardous reagents, ii) IP rights, geographical availability and animal welfare, iii) costs, analysis time, sample amount, competitiveness, iv) others.

The MCTT HCE™ EIT method makes use of the water soluble tetrazolium salt WST-1 to assess cell viability (instead of the MTT assay), which is spontaneously released into supernatant and therefore solvent extraction step is not necessary.

The model is produced and available in South Korea, and experiments were conducted in China showing appropriate responses to negative and positive controls as well as appropriate performance with the proficiency chemicals (Appendix 8 of the validation report sent to the PRP on 30 July 2018).

Finally, according to the information provided to the Peer Review Panel (PRP), the MCTT HCE™ EIT is not subject to intellectual property rights, and all components and reagents used for the MCTT HCE™ EIT are commercially available (Appendix 10 of the validation report sent to the PRP on 30 July 2018).

Evaluation criterion 2:

A detailed protocol for the similar or modified test method should be available.

The MCTT HCE™ is reconstructed using cultured primary human corneal epithelial cells from residual limbus tissues remaining after corneal transplantation (cf. validation report chapters III and IV.1 sent to the PRP on 30 July 2018). A detailed protocol of the MCTT HCE™ EIT is available and was considered to be adequate by the PRP (revised protocol 1.6 provided to the PRP on 9 July 2018).

Evaluation criterion 3:

Adherence to the essential test method components as described in paragraphs 4 to 21 of GD 216 should be demonstrated for the similar or modified test methods regarding i.e., the general conditions, the functional conditions and the procedural conditions.

Adherence to the essential test method components was considered to be adequate by the PRP

Evaluation criterion 4:

In addition, for modified test methods, the toxicological mechanisms and the relationship between the test method endpoint(s) with the biological effect as well as the toxicity of interest should be addressed, describing limitations of the test method.

Testing of the 30 Performance Standard (PS) chemicals with WST-1 showed similar outcomes as MTT (answers to PRP questions provided to the PRP on 9 July 2018). In addition, corrections of coloured and reducing chemicals interfering with WST-1 have been characterized and the necessary procedures clearly described (Appendix 3 distributed to the PRP on 4 June 2018). Based on the information provided by the test method developer, the PRP considered the use of WST-1 to be similar to the use of MTT. In addition, it is noted that the recently introduced test method Labcyte CORNEA-MODEL24 EIT to TG 492 makes use of WST-8.

Evaluation criterion 5:

At least the 30 recommended reference chemicals within GD 216 should be tested with the similar or modified test method according to recommendations of paragraphs 22, 23, 24 and 29 of GD 216, to demonstrate reliability and accuracy.

Notes: The exclusive use of the Reference Chemicals for the development/optimisation of new similar test methods should be avoided to the extent possible and the identity of all additional chemicals used for test method development (e.g., for setting the prediction model or exposure times) should be reported when submitting a PS-based validation study.

For test methods to be used by several independent laboratories, all of the 30 Reference Chemicals should be tested in at least three laboratories. In each laboratory, all Reference Chemicals should be tested in three independent runs performed with different tissue batches and at sufficiently spaced time points. Each run should consist of at least two concurrently tested tissue replicates.

In case Reference Chemicals does/do not meet the test acceptance criteria or is/are not acceptable for technical reasons or because they were obtained in a non-qualified run, a maximum number of two additional tests/runs for each Reference Chemical is admissible per laboratory ("re-testing"). Non-qualified tests should be documented and reported. Excess production of data and subsequent data selection are regarded as not appropriate.

The test method developer tested the 30 PS reference chemicals and an additional set of 130 chemicals that were used initially to develop the prediction model of the assay (including 19 overlapping PS reference chemicals). The overall dataset tested in the MCTT HCE™ EIT comprised 141 non-overlapping chemicals including liquids (87) and solids (54). The PRP considers the number and distribution of chemicals tested to be sufficient and adequate.

Evaluation criterion 6:

The reliability obtained with the reference chemicals, calculated as described in paragraph 25.1 and 25.2, should be equal to or better than the defined minimum target values for the similar or modified test method as specified in paragraphs 26 and 27 of GD 216 (within-laboratory reproducibility ≥ 90%, between-laboratory reproducibility ≥ 85%).

The PRP considered the reliability of the MCTT HCE™ EIT as obtained with the reference chemicals and the new proposed cut-offs (60% for solids, 35% for liquids) as provided to the PRP in the answers to PRP questions on 9 July 2018 and summarized below to be sufficient and adequate.

Table 1: Within- and Between- laboratory reproducibility of the MCTT HCE™ EIT based on the 30 reference chemicals recommended within the OECD GD 216 (2017)) (extract from table 16 of the revised PS-based validation report sent on 30 July 2018 to the PRP)

Performance Standard (PS) chemicals from GD 216 (2017)	WLR			BLR	Minimum required PS	
	BS	KCL	BTT		WLR	BLR
New proposed Prediction Model (liquid 35%/ solid 60%)	**96.7%** (29/30)	**90.0%** (27/30)	**90.0%** (27/30)	**93.3%** (28/30)	90%	85%

In addition, only one out of the 30 reference chemicals required more than two test repetitions in two of the three participating laboratories (dipropyl disulphide) due to borderline results to the old cut-off of 45%. However, with the new proposed cut-off, this chemical would no longer be considered borderline, and the new borderline chemicals were not re-tested leading to the performances

described here above. Based on this, it is considered that study quality criteria as described within the OECD GD 216 were met.

Evaluation criterion 7:

The predictive capacity obtained with the reference chemicals, calculated as described in paragraph 25.3, should be equal to or better than the defined minimum target values for the similar or modified test method as specified in paragraph 27 of GD 220 (sensitivity ≥ 90%, specificity ≥ 60%, accuracy ≥ 75%).

The predictive capacity obtained with the new proposed cut-offs (60% for solids, 35% for liquids) was considered to be sufficient and adequate by the PRP both for the PS reference chemicals and for the enlarged dataset of 141 chemicals. Below is a summary of the performances obtained.

Table 2: Performances of the MCTT HCE™ EIT based on the 30 reference chemicals recommended within the OECD GD 216 (2017) (extract from table 17 of the revised PS-based validation report sent on 30 July 2018 to the PRP)

	30 Reference Chemicals (GD 216, 2017)	**PS target values**
Sensitivity (n=15)	**92.6%**	≥ 90%
Specificity (n=15)	**60.7%**	≥ 60%
Accuracy (n=30)	**76.7%**	≥ 75%

Table 3: Performances of the MCTT HCE™ EIT based on the entire dataset of 141 non-overlapping tested chemicals (extract from table 15 of the revised PS-based validation report sent on 30 July 2018 to the PRP)

	Total	**Liquids**	**Solids**
Sensitivity	**98.8%** (n=80)	100% (n=49)	96.8% (n=31)
Specificity	**68.9%** (n=61)	63.2% (n=38)	78.3% (n=23)
Accuracy	**85.8%** (n=141)	83.9% (n=87)	88.9% (n=54)

Evaluation criterion 8:

The applicability domain of the new or modified test method should be defined.

A total of 111 chemicals with clear identification and *in vivo* classification have been tested in addition to the 30 reference chemicals, so that the characterization of the applicability domain of the MCTT HCE™ EIT was considered adequate and sufficient by the PRP.

Evaluation criterion 9:

All data from the PS-based validation study supporting the validity of the similar or modified test method should be obtained in accordance with the principles of Good Laboratory Practice (GLP).

According to the information provided to the PRP, the study was conducted according to GLP principles. In particular, two of the three participating laboratories are OECD GLP-accredited

contract research organizations from MFDS, Korea, and the third laboratory conducted the tests in the spirit of GLP.

Evaluation criterion 10:

Completeness of all data and documents supporting the assessment of the validity of the similar or modified test method.

The information provided by the test method developer was considered to be sufficient for the assessment of the similarity of the MCTT HCE™ EIT.

Evaluation criterion 11:

PS-based validation study management and conduct.

The PRP considered the information provided on the study management conduct to be adequate and sufficient.

Evaluation criterion 12:

Other considerations.

12.1. Quality control procedures for lot release

The PRP considered the quality control procedures for lot release sufficient provided that the tissue developer continues to assess the barrier function of the MCTT HCE™ tissue batches by calculating the ET_{50} by interpolating between two exposure times resulting in a viability above and a viability below 50%. This is due to the fact that the correlation between log-transformed (Log10) time of exposure and cell viability may not always be linear. Furthermore it is suggested that the tissue developer continues to report the figures based on cell viability rather than OD.

12.2. Audit of tissue production

Based on the information provided to the PRP, the manufacturing of the MCTT HCE™ tissues has been independently audited within the framework of an ISO 9001:2005 certification.

12.3. Colour interfering chemicals

The PRP is of the opinion that assessment of the colour interference with the WST-1 was appropriately conducted and reported (Appendix12 of revised validation report sent to the PRP on 30 July 2018).

Evaluation criterion 13:

All data should adequately support the peer review assessment that the proposed test method is structurally and mechanistically similar to the validated reference method, and demonstrate sufficient reliability and relevance for the proposed specific testing purpose i.e., that the proposed similar or modified test method is scientifically valid.

The data assessed by the peer-review panel supports the scientific similarity of the MCTT HCE™ EIT to the validated reference methods both in terms of the essential test method components and of assay performance regarding its reproducibility and predictive capacity as described within the GD 216.